LIVE CELL CONSTRUCTS FOR MILK PRODUCTION AND METHODS USING THE SAME

FIELD OF THE INVENTION

This invention relates to live cell constructs and methods using the same for *in vitro* and/or *ex vivo* production of milk from cultured mammary cells.

BACKGROUND OF THE INVENTION

Milk is a staple of the human diet, both during infancy and throughout life. The American Academy of Pediatrics and World Health Organization recommend that infants be exclusively breastfed for the first 6 months of life, and consumption of dairy beyond infancy is a mainstay of human nutrition, representing a 700 billion dollar industry worldwide. However, lactation is a physiologically demanding and metabolically intensive process that can present biological and practical challenges for breastfeeding mothers, and milk production is associated with environmental, social, and animal welfare impacts in agricultural contexts.

The possibility of using mammalian cell culture to produce food has gained increasing interest in recent years, with the development of several successful prototypes of meat and sea food products from cultured muscle and fat cells (Stephens et al. 2018 *Trends Food Sci Technol.* 78:155-166). Additionally, efforts are underway to commercialize the production of egg and milk proteins using microbial expression systems. However, this fermentation-based process relies on the genetically engineered expression and purification of individual components and is unable to reproduce the full molecular profile of milk or dairy.

The present invention overcomes shortcomings in the art by providing live cell constructs and methods using the same for *in vitro* and/or *ex vivo* production of milk from cultured mammary cells.

SUMMARY OF THE INVENTION

The present invention is based, in part, on the development of live cell constructs comprising mammary cells that compartmentalize feeding of the cells and secretion of milk.

Thus, one aspect of the invention relates to a live cell construct comprising, a scaffold having a top surface and a bottom surface; and a continuous monolayer of (a) live primary mammary epithelial cells, (b) a mixed population of live primary mammary epithelial cells,

EXHIBIT C

mammary myoepithelial cells and mammary progenitor cells, and/or (c) live immortalized mammary epithelial cells on the top surface of the scaffold, the continuous monolayer of (a) live primary mammary epithelial cells, (b) mixed population of live primary mammary epithelial cells mammary myoepithelial cells and mammary progenitor cells, and/or (c) immortalized mammary epithelial cells having an apical surface and a basal surface (e.g., the cells form a polarized and confluent cell monolayer), wherein the construct comprises an apical compartment above and adjacent to the apical surface of the continuous monolayer of the (a) live primary mammary epithelial cells, the (b) mixed population of live primary mammary epithelial cells, mammary myoepithelial cell and mammary progenitor cells, and/or the (c) immortalized mammary epithelial cells and a basal compartment below and adjacent to the bottom surface of the scaffold.

Another aspect of the invention provides a method of producing milk in culture, the method comprising culturing the live cell construct of the present invention, thereby producing milk in culture.

An additional aspect of the invention provides a method of making a live cell construct for producing milk in culture, the method comprising (a) isolating primary mammary epithelial cells, myoepithelial cells and/or mammary progenitor cells from mammary explants from mammary tissue, to produce isolated mammary epithelial cells, myoepithelial cells and mammary progenitor cells; (b) culturing the isolated primary mammary epithelial cells, myoepithelial cells and mammary progenitor cells to produce a mixed population of primary mammary epithelial cells, mammary myoepithelial cells and mammary progenitor cells; (c) cultivating the mixed population of (b) on a scaffold, the scaffold having an upper surface and lower surface, to produce a polarized, continuous (i.e., confluent) monolayer of primary mammary epithelial cells, myoepithelial cells and mammary progenitor cells of the mixed population on the upper surface of the scaffold, wherein the polarized, continuous monolayer comprises an apical surface and a basal surface, thereby producing a live cell construct for producing milk in culture.

A further aspect of the present invention relates to a method of making a live cell construct for producing milk in culture, the method comprising: a) isolating primary mammary epithelial cells, myoepithelial cells, and/or mammary progenitor cells from mammary explants from mammary tissue (e.g., breast, udder, teat tissue), to produce isolated mammary epithelial cells, myoepithelial cells, and/or mammary progenitor cells; (b) culturing the isolated primary mammary epithelial cells, myoepithelial cells, and/or mammary

progenitor cells to produce a mixed population of primary mammary epithelial cells, mammary myoepithelial cells and mammary progenitor cells; (c) sorting the mixed population of primary mammary epithelial cells, myoepithelial cells, and/or mammary progenitor cells to produce a population of primary mammary epithelial cells; and (d) cultivating the population of primary mammary epithelial cells on a scaffold, the scaffold having an upper surface and lower surface, to produce a polarized, continuous (i.e., confluent) monolayer of primary mammary epithelial cells on the upper surface of the scaffold, wherein the polarized, continuous monolayer comprises an apical surface and a basal surface, thereby producing a live cell construct for producing milk in culture.

Another aspect of the present invention relates to a method of making a live cell construct for producing milk in culture, the method comprising (a) culturing immortalized mammary epithelial cells to produce increased numbers of immortalized mammary epithelial cells; (b) cultivating the immortalized mammary epithelial cells of (a) on a scaffold, the scaffold having an upper surface and lower surface, to produce a polarized, continuous (i.e., confluent) monolayer of immortalized mammary epithelial cells on the upper surface of the scaffold, wherein the polarized, continuous monolayer comprises an apical surface and a basal surface, thereby producing a live cell construct for producing milk in culture.

Another aspect of the present invention relates a method of producing milk in culture comprising, culturing a live cell construct comprising (a) a scaffold comprising an upper surface and a lower surface and a continuous (i.e., confluent) polarized monolayer of live primary mammary epithelial cells, a continuous polarized monolayer of a mixed population of live primary mammary epithelial cells, mammary myoepithelial cells and mammary progenitor cells, and/or a continuous polarized monolayer of live immortalized mammary epithelial cells having an apical surface and a basal surface, wherein the continuous polarized monolayer of live primary mammary epithelial cells, the continuous polarized monolayer of the mixed population of live primary mammary epithelial cells, mammary myoepithelial cells and mammary progenitor cells and/or the continuous polarized monolayer of live immortalized mammary epithelial cells are located on the upper surface of scaffold, (b) a basal compartment and an apical compartment, wherein the lower surface of the scaffold is adjacent to the basal compartment and the apical surface of the monolayer of live primary mammary epithelial cells, the monolayer of the mixed population of live primary mammary epithelial cells, mammary myoepithelial cells and mammary progenitor cells, and/or the monolayer of live immortalized mammary epithelial cells is adjacent to the apical

compartment, wherein the monolayer of live primary epithelial mammary cells, the live primary epithelial mammary cells of the monolayer of the mixed population of live primary mammary epithelial cells, mammary myoepithelial cells and mammary progenitor cells, or the monolayer of immortalized mammary epithelial cells excretes milk through its apical surface into the apical compartment, thereby producing milk in culture.

A further aspect of the present invention relates to a method of producing a modified primary mammary epithelial cell or a immortalized mammary epithelial cell, wherein the method comprises introducing into the cell: (a) a polynucleotide encoding a prolactin receptor comprising a modified intracellular signaling domain, optionally wherein the prolactin receptor comprises a truncation wherein position 154 of exon 10 has been spliced to the 3' sequence of exon 11; (b) a polynucleotide encoding a chimeric prolactin receptor that binds to a ligand, which is capable of activating milk synthesis in the absence of prolactin; (c) a polynucleotide encoding a constitutively or conditionally active prolactin receptor protein, optionally wherein the polynucleotide encodes a constitutively active human prolactin receptor protein comprising a deletion of amino acids 9 through 187; (d) a polynucleotide encoding a modified (recombinant) effector of a prolactin protein comprising (i) a JAK2 tyrosine kinase domain fused to a STAT5 tyrosine kinase domain; and/or (ii) a prolactin receptor intracellular domain fused to a JAK2 tyrosine kinase domain; (e) a loss of function mutation into a circadian related gene *PER2* (period circadian protein homolog 2); and/or (f) a polynucleotide encoding one or more glucose transporter genes GLUT1 and/or GLUT12, thereby increasing the rate of nutrient uptake at the basal surface of a monolayer of cells of the modified primary mammary epithelial cell or immortalized mammary epithelial cell.

These and other aspects of the invention are set forth in more detail in the description of the invention below.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 shows an example of the collection of milk for nutritional use from mammary epithelial cells grown as a confluent monolayer in a compartmentalizing culture apparatus in which either fresh or recycled media is provided to the basal compartment and milk is collected from the apical compartment. TEER, transepithelial electrical resistance.

FIG. 2 shows an example of polarized absorption of nutrients and secretion of milk across a confluent monolayer of mammary epithelial cells anchored to a scaffold at the basal surface.

FIG. 3 shows an example micropatterned scaffold provides increased surface area for the compartmentalized absorption of nutrients and secretion of milk by a confluent monolayer of mammary epithelial cells.

FIG. 4 shows three examples of a hollow fiber bioreactor depicted as a bundle of capillary tubes (top), which can support mammary epithelial cells lining either the external (top and lower left) or internal (lower right) surface of the capillaries, providing directional and compartmentalized absorption of nutrients and secretion of milk.

DETAILED DESCRIPTION OF THE INVENTION

The present invention is explained in greater detail below. This description is not intended to be a detailed catalog of all the different ways in which the invention may be implemented, or all the features that may be added to the instant invention. For example, features illustrated with respect to one embodiment may be incorporated into other embodiments, and features illustrated with respect to a particular embodiment may be deleted from that embodiment. In addition, numerous variations and additions to the various embodiments suggested herein will be apparent to those skilled in the art in light of the instant disclosure which do not depart from the instant invention. Hence, the following specification is intended to illustrate some particular embodiments of the invention, and not to exhaustively specify all permutations, combinations and variations thereof.

Unless the context indicates otherwise, it is specifically intended that the various features of the invention described herein can be used in any combination. Moreover, the present invention also contemplates that in some embodiments of the invention, any feature or combination of features set forth herein can be excluded or omitted. To illustrate, if the specification states that a complex comprises components A, B and C, it is specifically intended that any of A, B or C, or a combination thereof, can be omitted and disclaimed singularly or in any combination.

Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. The terminology used in the description of the invention herein is for the purpose of describing particular embodiments only and is not intended to be limiting of the invention.

Nucleotide sequences are presented herein by single strand only, in the 5' to 3' direction, from left to right, unless specifically indicated otherwise. Nucleotides and amino acids are represented herein in the manner recommended by the IUPAC-IUB Biochemical

Nomenclature Commission, or (for amino acids) by either the one-letter code, or the three letter code, both in accordance with 37 C.F.R. §1.822 and established usage.

Except as otherwise indicated, standard methods known to those skilled in the art may be used for production of recombinant and synthetic polypeptides, antibodies or antigen-binding fragments thereof, manipulation of nucleic acid sequences, production of transformed cells, the construction of viral vector constructs, and transiently and stably transfected packaging cells. Such techniques are known to those skilled in the art. *See, e.g.,* Sambrook *et al.*, Molecular Cloning: A Laboratory Manual 2nd Ed. (Cold Spring Harbor, NY, 1989); F. M. Ausubel *et al.* Current Protocols In Molecular Biology (Green Publishing Associates, Inc. and John Wiley & Sons, Inc., New York).

All publications, patent applications, patents, nucleotide sequences, amino acid sequences and other references mentioned herein are incorporated by reference in their entireties for the teachings relevant to the sentence and/or paragraph in which the reference is presented.

Definitions

As used in the description of the invention and the appended claims, the singular forms "a," "an" and "the" are intended to include the plural forms as well, unless the context clearly indicates otherwise.

As used herein, "and/or" refers to and encompasses any and all possible combinations of one or more of the associated listed items, as well as the lack of combinations when interpreted in the alternative ("or").

Moreover, the present invention also contemplates that in some embodiments of the invention, any feature or combination of features set forth herein can be excluded or omitted.

Furthermore, the term "about," as used herein when referring to a measurable value such as an amount of a compound or agent of this invention, dose, time, temperature, and the like, is meant to encompass variations of ± 10%, ± 5%, ± 1%, ± 0.5%, or even ± 0.1% of the specified amount.

As used herein, the transitional phrase "consisting essentially of" is to be interpreted as encompassing the recited materials or steps and those that do not materially affect the basic and novel characteristic(s) of the claimed invention. Thus, the term "consisting essentially of" as used herein should not be interpreted as equivalent to "comprising."

As used herein, the term "polypeptide" encompasses both peptides and proteins, unless indicated otherwise.

The term "materially altered" (or grammatical equivalents, e.g., "modified") as applied to polynucleotides and/or polypeptides of the invention, refers to a polynucleotide and/or polypeptide that is substantially modified from its native form in composition and/or genomic locus by deliberate human intervention

As used herein, by "isolate" (or grammatical equivalents, e.g., "extract") a product, it is meant that the product is at least partially separated from at least some of the other components in the starting material.

By "substantially retain" a property, it is meant that at least about 75%, 85%, 90%, 95%, 97%, 98%, 99% or 100% of the property (*e.g.*, activity or other measurable characteristic) is retained.

The term "polarized" as used herein in reference to cells and/or monolayers of cells refers to a spatial status of the cell wherein there are two distinct surfaces of the cell, e.g., an apical surface and a basal surface, which may be different (e.g., may comprise different surface and/or transmembrane receptors and/or other structures). Individual polarized cells in a continuous monolayer may have similarly-oriented apical surfaces and basal surfaces, and may have communicative structures between individual cells (e.g., tight junctions) to allow cross communication between individual cells and to create separation (e.g., compartmentalization) of the apical compartment (e.g., the lumen above and adjacent to the apical surface) and basal compartment (e.g., the lumen below and adjacent to the basal surface).

The term "lactogenic" as used herein refers to the ability to stimulate production and/or secretion of milk. A lactogenic product may be a gene, protein (e.g., prolactin), or other natural and/or synthetic product. A culture medium comprising lactogenic properties (e.g., comprising prolactin, thereby stimulating production of milk by cells in contact with the culture medium) may be referred to as a "lactogenic culture medium."

As used herein, the term "food grade" refers to materials considered non-toxic and safe for consumption (e.g., human and/or other animal consumption), e.g., as regulated by standards set by the U.S. Food and Drug Administration.

Live Cell Constructs

The present invention relates to live cell constructs, methods of making the same, and methods of using the same for *in vitro* and/or *ex vivo* production of milk from cultured mammary cells. Milk is a complex macromolecular secretion composed of proteins, lipids, and carbohydrates produced by epithelial cells that line the internal compartment of the mammary gland. Mammary epithelial cells in culture have been previously demonstrated to display organization and behavior similar to that observed *in vivo* (Arevalo et al. 2016 *Am J Physiol Cell Physiol.* 310(5):C348-356; Chen et al. 2019 *Curr Protoc Cell Biol.* 82(1):e65). In particular, when grown on an appropriate extracellular matrix and stimulated with prolactin, cultured mammary epithelial cells organize into polarized structures and secrete milk components (Blatchford et al. 1999 *Animal Cell Technology: Basic & Applied Aspects* 10:141-145). However, as previous studies have been focused on basic and biomedical research, nutritional applications of *in vitro* milk production remain unexplored and no attempt has been made to collect the milk separately from the medium in which the cells are grown.

Thus, one aspect of the invention relates to a live cell construct comprising, a scaffold having a top surface and a bottom surface; and a continuous monolayer of (a) live primary mammary epithelial cells, (b) a mixed population of live primary mammary epithelial cells, mammary myoepithelial cells and mammary progenitor cells, and/or (c) live immortalized mammary epithelial cells on the top surface of the scaffold, the continuous monolayer of (a) live primary mammary epithelial cells, (b) mixed population of live primary mammary epithelial cells mammary myoepithelial cells and mammary progenitor cells, and/or (c) immortalized mammary epithelial cells having an apical surface and a basal surface (e.g., the cells form a polarized and confluent cell monolayer), wherein the construct comprises an apical compartment above and adjacent to the apical surface of the continuous monolayer of the (a) live primary mammary epithelial cells, the (b) mixed population of live primary mammary epithelial cells, mammary myoepithelial cell and mammary progenitor cells, and/or the (c) immortalized mammary epithelial cells and a basal compartment below and adjacent to the bottom surface of the scaffold.

A live primary culture of mammary gland tissue may comprise milk-producing mammary epithelial cells, contractile myoepithelial cells, and/or progenitor cells that can give rise to both mammary epithelial and mammary contractile myoepithelial cells. Mammary epithelial cells are the only cells that produce milk. The live primary mammary epithelial

cells, the mixed population of live primary mammary epithelial cells, mammary myoepithelial cells and mammary progenitor cells, and/or the immortalized mammary epithelial cells may be from any mammal, e.g., a primate (e.g., chimpanzee, orangutan, gorilla, monkey (e.g., Old World, New World), lemur, human), a dog, a cat, a rabbit, a mouse, a rat, a horse, a cow, a goat, a sheep, an ox (e.g., *Bos* spp.), a pig, a deer, a musk deer, a bovid, a whale, a dolphin, a hippopotamus, an elephant, a rhinoceros, a giraffe, a zebra, a lion, a cheetah, a tiger, a panda, a red panda, and an otter. In some embodiments, the live primary mammary epithelial cells, the mixed population of live primary mammary epithelial cells, mammary myoepithelial cells and mammary progenitor cells, and/or the immortalized mammary epithelial cells may be from an endangered species, e.g., an endangered mammal. In some embodiments, the live primary mammary epithelial cells, the mixed population of live primary mammary epithelial cells, mammary myoepithelial cells and mammary progenitor cells, and/or the immortalized mammary epithelial cells may be from a human. In some embodiments, the live primary mammary epithelial cells, the mixed population of live primary mammary epithelial cells, mammary myoepithelial cells and mammary progenitor cells, and/or the immortalized mammary epithelial cells may be from a bovid (e.g., a cow).

In some embodiments, milk produced by the primary mammary epithelial cells (e.g., primary mammary epithelial cells from the isolated live primary mammary epithelial cells and/or the primary mammary epithelial cells from the mixed population of live primary mammary epithelial cells, mammary myoepithelial cells and/or mammary progenitor cells) or the immortalized mammary epithelial cells may be excreted through the apical surface of the cells into the apical compartment.

In some embodiments, a basal compartment may comprise a basal culture medium and the basal culture medium may be in contact with the basal surface of the live primary mammary epithelial cells, the mixed population of live primary mammary epithelial cells, mammary myoepithelial cells and mammary progenitor cells, and/or the immortalized mammary epithelial cells.

In some embodiments, the basal culture medium of the present invention may comprise a carbon source, a chemical buffering system, one or more essential amino acids, one or more vitamins and/or cofactors, and one or more inorganic salts.

In some embodiments, the basal culture medium may comprise a carbon source in an amount from about 1 g/L to about 15 g/L of basal culture medium (e.g., about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14 or 15 g/L or any value or range therein), or about 1, 2, 3, 4, 5 or 6

g/L to about 7, 8, 9, or 10, 11, 12, 13, 14 or 15 g/L of the basal culture medium. Non-limiting examples of a carbon source include glucose and/or pyruvate. For example, in some embodiments, the basal culture medium may comprise glucose in an amount from about 1g/L to about 12 g/L of basal culture medium, e.g., about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, or 12 g/L or any value or range therein. In some embodiments, the basal culture medium may comprise glucose in an amount from about 1 g/L to about 6 g/L, about 4 g/L to about 12 g/L, about 2.5 g/L to about 10.5 g/L, about 1.5 g/L to about 11.5 g/L, or about 2g/L to about 10 g/L of basal culture medium. In some embodiments, the basal culture medium may comprise glucose in an amount from about 1, 2, 3, or 4 g/L to about 5, 6, 7, 8, 9, 10, 11, or 12 g/L or about 1, 2, 3, 4, 5, or 6 g/L to about 7, 8, 9, 10, 11, or 12 g/L. In some embodiments, the basal culture medium may comprise pyruvate in an amount from about 5 g/L to about 15 g/L of basal culture medium, e.g., about 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, or 15 g/L or any value or range therein. In some embodiments, the basal culture medium may comprise pyruvate in an amount from about 5 g/L to about 14.5 g/L, about 10g/L to about 15 g/L, about 7.5 g/L to about 10.5 g/L, about 5.5g/L to about 14.5 g/L, or about 8g/L to about 10 g/L of basal culture medium. In some embodiments, the basal culture medium may comprise pyruvate in an amount from about 5, 6, 7, or 8 g/L to about 9, 10, 11, 12, 13, 14 or 15 g/L or about 5, 6, 7, 8, 9, or 10 g/L to about 11, 12, 13, 14 or 15 g/L.

In some embodiments, the basal culture medium may comprise a chemical buffering system in an amount from about 1g/L to about 4 g/L (e.g., about 1, 1.5, 2, 2.5, 3, 3.5, or 4 g/L or any value or range therein) of basal culture medium or about 10 mM to about 25 mM (e.g., about 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, or 25 mM or any value or range therein). In some embodiments, the chemical buffering system may include, but is not limited to, sodium bicarbonate and/or 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES). For example, in some embodiments, the basal culture medium may comprise sodium bicarbonate in an amount from about 1g/L to about 4 g/L of basal culture medium, e.g., about 1, 1.5, 2, 2.5, 3, 3.5, or 4 g/L or any value or range therein. In some embodiments, the basal culture medium may comprise sodium bicarbonate in an amount from about 1 g/L to about 3.75 g/L, about 1.25 g/L to about 4 g/L, about 2.5 g/L to about 3 g/L, about 1.5 g/L to about 4 g/L, or about 2g/L to about 3.5 g/L of basal culture medium. In some embodiments, the basal culture medium may comprise HEPES in an amount from about 10 mM to about 25 mM, e.g., about 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, or 25 mM or any value or range therein. In some embodiments, the basal culture medium may

comprise HEPES in an amount from about 11 mM to about 25 mM, about 10 mM to about 20 mM, about 12.5 mM to about 22.5 mM, about 15 mM to about 20.75 mM, or about 10 mM to about 20 mM.

In some embodiments, the basal culture medium may comprise one or more essential amino acids in an amount from about 0.5 mM to about 5 mM (e.g., about 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5, or 5 mM or any value or range therein) or about 0.5, 1, 1.5, 2 mM to about 2.5, 3, 3.5, 4, 4.5, or 5 mM. In some embodiments, the one or more essential amino acids may be, for example, arginine and/or cysteine. For example, in some embodiments, the basal culture medium may comprise arginine in an amount from about 0.5 mM to about 5 mM, e.g., about 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5, or 5 mM or any value or range therein. In some embodiments, the basal culture medium may comprise arginine in an amount from about 0.5 mM to about 4.75 mM, about 2 mM to about 3.5 mM, about 0.5 mM to about 3.5 mM, about 1 mM to about 5 mM, or about 3.5 mM to about 5 mM. For example, in some embodiments, the basal culture medium may comprise cysteine in an amount from about 0.5 mM to about 5 mM, e.g., about 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5, or 5 mM or any value or range therein. In some embodiments, the basal culture medium may comprise cysteine in an amount from about 0.5 mM to about 4.75 mM, about 2 mM to about 3.5 mM, about 0.5 mM to about 3.5 mM, about 1 mM to about 5 mM, or about 3.5 mM to about 5 mM.

In some embodiments, the basal culture medium may comprise one or more vitamins and/or cofactors in an amount from about 0.01 µM to about 50 µM (e.g., about 0.01, 0.02, 0.03, 0.04, 0.05, 0.06, 0.07, 0.08, 0.09, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1, 1.1, 1.2, 1.3, 1.4, 1.5, 1.6, 1.7, 1.8, 1.9, 2, 2.1, 2.2, 2.3, 2.4, 2.5, 3, 4, 5, 6, 7, 8, 9, 10, 12.5, 15, 17.5, 20, 25, 30, 35, 40, 45, 46, 47, 48, 49, 49.025, 49.05, 49.075, or 50 µM or any value or range therein) or about 0.01, 0.02, 0.03, 0.04, 0.05, 0.06, 0.07, 0.08, 0.09, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, or 0.9 µM to about 1, 1.1, 1.2, 1.3, 1.4, 1.5, 1.6, 1.7, 1.8, 1.9, 2, 2.1, 2.2, 2.3, 2.4, 2.5, 3, 4, 5, 6 µM or about 0.02, 0.025, 0.05, 0.075, 1, 1.5, 2, 3, 4, 5, 6, 7, 8, 9, 10 µM to about 12.5, 15, 17.5, 20, 25, 30, 35, 40, 45, 46, 47, 48, 49, 49.025, 49.05, 49.075, or 50 µM. In some embodiments, one or more vitamins and/or cofactors may include, but are not limited to, thiamine and/or riboflavin. For example, in some embodiments, the basal culture medium may comprise thiamine in an amount from about 0.025 µM to about 50 µM, e.g., about 0.025, 0.05, 0.075, 1, 1.5, 2, 3, 4, 5, 6, 7, 8, 9, 10, 12.5, 15, 17.5, 20, 25, 30, 35, 40, 45, 46, 47, 48, 49, 49.025, 49.05, 49.075, or 50 µM or any value or range therein. In some embodiments, the basal culture medium may comprise thiamine in an amount from about

0.025 μM to about 45.075 μM, about 1 μM to about 40 μM, about 5 μM to about 35.075 μM, about 10 μM to about 50 μM, or about 0.05 μM to about 45.5 μM. In some embodiments, the basal culture medium may comprise riboflavin in an amount from about 0.01 μM to about 3 μM, e.g., about 0.01, 0.02, 0.03, 0.04, 0.05, 0.06, 0.07, 0.08, 0.09, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1, 1.1, 1.2, 1.3, 1.4, 1.5, 1.6, 1.7, 1.8, 1.9, 2, 2.1, 2.2, 2.3, 2.4, 2.5, 2.6, 2.7, 2.8, 2.9, or 3 μM or any value or range therein. In some embodiments, the basal culture medium may comprise riboflavin in an amount from about 0.01 μM to about 2.05 μM, about 1 μM to about 2.95 μM, about 0.05 μM to about 3 μM, about 0.08 μM to about 1.55 μM, or about 0.05 μM to about 2.9 μM.

In some embodiments, the basal culture medium may comprise one or more inorganic salts in an amount from about 100 mg/L to about 150 mg/L of basal culture medium (e.g., about 100, 105, 110, 115, 120, 125, 130, 135, 140, 145, or 150 mg/L or any value or range therein) or about 100 mg/L to about 150 mg/L of basal culture medium (e.g., about 100, 105, 110, 115, 120, 125, 130, 135, 140, 145, or 150 mg/L or any value or range therein). In some embodiments, one or more inorganic salts may include, but are not limited to, calcium and/or magnesium. For example, in some embodiments, the basal culture medium may comprise calcium in an amount from about 100 mg/L to about 150 mg/L of basal culture medium, e.g., about 100, 105, 110, 115, 120, 125, 130, 135, 140, 145, or 150 mg/L or any value or range therein. In some embodiments, the basal culture medium may comprise arginine in an amount from about 100 mg/L to about 125 mg/L, about 105 mg/L to about 150 mg/L, about 120 mg/L to about 130 mg/L, or about 100 mg/L to about 145 mg/L of basal culture medium. In some embodiments, the basal culture medium may comprise magnesium in an amount from about 0.01 mM to about 1 mM, e.g., about 0.01, 0.02, 0.03, 0.04, 0.05, 0.06, 0.07, 0.08, 0.09, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 0.91, 0.92, 0.93, 0.94, 0.95, 0.96, 0.97, 0.98, 0.99, or 1 mM or any value or range therein. In some embodiments, the basal culture medium may comprise magnesium in an amount from about 0.05 mM to about 1 mM, about 0.01 mM to about 0.78 mM, about 0.5 mM to about 1 mM, about 0.03 mM to about 0.75 mM, or about 0.25 mM to about 0.95 mM.

In some embodiments, the carbon source, chemical buffering system, one or more essential amino acids, one or more vitamins and/or cofactors, and/or one or more inorganic salts may be food grade. In some embodiments, the basal culture medium may be lactogenic culture medium, e.g., the basal culture medium may further comprise prolactin (e.g., mammalian prolactin, e.g., human prolactin). For example, in some embodiments, the basal

culture medium may comprise prolactin (or prolactin may be added) in an amount from about 20 ng/mL to about 200 ng/L of basal culture medium, e.g., about 20, 30, 40, 50, 60, 70, 80, 90, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190, or 200 ng/mL or any value or range therein. In some embodiments, the basal culture medium may comprise prolactin (or prolactin may be added) in an amount from about 20 ng/mL to about 195 ng/mL, about 50 ng/mL to about 150 ng/mL, about 25 ng/mL to about 175 ng/mL, about 45 ng/mL to about 200 ng/mL, or about 75 ng/mL to about 190 ng/mL of basal culture medium. In some embodiments, the basal culture medium may further comprise other factors to improve efficiency, including, but not limited to, insulin, an epidermal growth factor, and/or a hydrocortisone.

In some embodiments, the scaffold of the present invention may be fabricated as a 2-dimensional surface, a 3-dimensional micropatterned surface, and/or as a cylindrical structure that can be assembled into bundles. Non-limiting examples of a 2-dimensional surface scaffold include a transwell filter. Non-limiting examples of a 3-dimensional micropatterned surface include a microstructured bioreactor, a decellularized tissue (e.g, a decellularized mammary gland) and/or a cylindrical structure that can be assembled into bundles (e.g., a hollow fiber bioreactor). In some embodiments, the scaffold of the present invention may be porous.

In some embodiments, the top surface of the scaffold may be coated with one or more extracellular matrix proteins. Non-limiting examples of extracellular matrix proteins include collagen, laminin, entactin, tenascin, and/or fibronectin. In some embodiments, the scaffold may comprise a natural polymer, a biocompatible synthetic polymer, a synthetic peptide, and/or a composite derived from any combination thereof. In some embodiments, a natural polymer useful with this invention may include, but is not limited to, collagen, chitosan, cellulose, agarose, alginate, gelatin, elastin, heparan sulfate, chondroitin sulfate, keratan sulfate, and/or hyaluronic acid. In some embodiments, a biocompatible synthetic polymer useful with this invention may include, but is not limited to, polysulfone, polyvinylidene fluoride, polyethylene co-vinyl acetate, polyvinyl alcohol, sodium polyacrylate, an acrylate polymer, and/or polyethylene glycol.

Methods

The present invention further provides methods of making a live cell construct, methods of producing milk in culture, and/or methods of producing a modified primary

mammary epithelial cell or an immortalized mammary epithelial cell, e.g., for use in the present invention.

Thus, in some embodiments, the present invention provides a method of producing milk in culture, the method comprising culturing the live cell construct of the present invention, thereby producing milk in culture.

In some embodiments, the present invention provides a method of making a live cell construct for producing milk in culture, the method comprising (a) isolating primary mammary epithelial cells, myoepithelial cells and mammary progenitor cells from mammary explants from mammary tissue (e.g., breast, udder, teat tissue), to produce isolated mammary epithelial cells, myoepithelial cells and mammary progenitor cells; (b) culturing the isolated primary mammary epithelial cells, myoepithelial cells and mammary progenitor cells to produce a mixed population of primary mammary epithelial cells, mammary myoepithelial cells and mammary progenitor cells; (c) cultivating the mixed population of (b) on a scaffold, the scaffold having an upper surface and lower surface, to produce a polarized, continuous (i.e., confluent) monolayer of primary mammary epithelial cells, myoepithelial cells and mammary progenitor cells of the mixed population on the upper surface of the scaffold, wherein the polarized, continuous monolayer comprises an apical surface and a basal surface, thereby producing a live cell construct for producing milk in culture.

In some embodiments, the present invention provides a method of making a live cell construct for producing milk in culture, the method comprising: a) isolating primary mammary epithelial cells, myoepithelial cells, and/or mammary progenitor cells from mammary explants from mammary tissue (e.g., breast, udder, teat tissue), to produce isolated mammary epithelial cells, myoepithelial cells, and/or mammary progenitor cells; (b) culturing the isolated primary mammary epithelial cells, myoepithelial cells, and/or mammary progenitor cells to produce a mixed population of primary mammary epithelial cells, mammary myoepithelial cells and mammary progenitor cells; (c) sorting the mixed population of primary mammary epithelial cells, myoepithelial cells, and/or mammary progenitor cells (e.g., selecting the primary mammary epithelial cells) to produce a population of primary mammary epithelial cells; and (d) cultivating the population of primary mammary epithelial on a scaffold, the scaffold having an upper surface and lower surface, to produce a polarized, continuous (i.e., confluent) monolayer of primary mammary epithelial cells on the upper surface of the scaffold, wherein the polarized, continuous monolayer

comprises an apical surface and a basal surface, thereby producing a live cell construct for producing milk in culture.

In some embodiments, the present invention provides a method of making a live cell construct for producing milk in culture, the method comprising (a) culturing immortalized mammary epithelial cells to produce increased numbers of immortalized mammary epithelial cells; (b) cultivating the immortalized mammary epithelial cells of (a) on a scaffold, the scaffold having an upper surface and lower surface, to produce a polarized, continuous (i.e., confluent) monolayer of immortalized mammary epithelial cells on the upper surface of the scaffold, wherein the polarized, continuous monolayer comprises an apical surface and a basal surface, thereby producing a live cell construct for producing milk in culture.

In some embodiments, mammary tissue may be from breast tissue, udder tissue, and/or teat tissue of a mammal. Mammary tissue may be from any mammal, e.g., a primate (e.g., chimpanzee, orangutan, gorilla, monkey (e.g., Old World, New World), lemur, human), a dog, a cat, a rabbit, a mouse, a rat, a horse, a cow, a goat, a sheep, an ox (e.g., *Bos* spp.), a pig, a deer, a musk deer, a bovid, a whale, a dolphin, a hippopotamus, an elephant, a rhinoceros, a giraffe, a zebra, a lion, a cheetah, a tiger, a panda, a red panda, and an otter. In some embodiments, the mammary tissue may be from an endangered species, e.g., an endangered mammal. In some embodiments, the mammary tissue may be from a human. In some embodiments, the mammary tissue may be from a bovid (e.g., a cow).

In some embodiments, the culturing and/or cultivating is carried out at a temperature of about 35°C to about 39°C (e.g., a temperature of about 35°C, 35.5°C, 36°C, 36.5°C, 37°C, 37.5°C, 38°C, 38.5°C or about 39°C, or any value or range therein, e.g., about 35°C to about 38°C, about 36°C to about 39°C, about 36.5°C to about 39°C, about 36.5°C to about 37.5°C, or about 36.5°C to about 38°C). In some embodiments, methods of the present invention may further comprise wherein the culturing is carried out at a temperature of about 37°C.

In some embodiments, the culturing and/or cultivating is carried out at an atmospheric concentration of CO_2 of about 4% to about 6%, e.g., an atmospheric concentration of CO_2 of about 4%, 4.25%, 4.5%, 4.75%, 5%, 5.25%, 5.5%, 5.75%, or 6% or any value or range therein, e.g., about 4% to about 5.5%, about 4.5% to about 6%, about 4.5% to about 5.5%, or about 5% to about 6%). In some embodiments, methods of the present invention may further comprise wherein the culturing is carried out at an atmospheric concentration of CO_2 of about 5%.

In some embodiments, the culturing and/or cultivating may comprise culturing and/or cultivating in a culture medium that is exchanged about every day to about every 10 days (e.g., every 1 day, every 2 days, every 3 days, every 4 days, every 5 days, every 6 days, every 7 days, every 8 days, every 9 days, every 10 days, or any value or range therein, e.g., about every day to every 3 days, about every 3 days to every 10 days, about every 2 days to every 5 days). In some embodiments, the culturing and/or cultivating may further comprise culturing in a culture medium that is exchanged about every day to about every few hours to about every 10 days, e.g., about every 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, or 24 hours to about every 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 days or any value or range therein. For example, in some embodiments, the culturing and/or cultivating may further comprise culturing and/or cultivating in a culture medium that is exchanged about every 12 hours to about every 10 days, about every 10 hours to about every 5 days, or about every 5 hours to about every 3 days.

In some embodiments, the monolayer of the live cell construct made by the methods of the invention for producing milk in culture may be adjacent to the upper surface of the scaffold.

In some embodiments, the live cell construct made by the methods of the invention for producing milk in culture may further comprise an apical compartment that is adjacent to the apical surface of the monolayer.

In some embodiments, the live cell construct made by the methods of the invention for producing milk in culture may comprise a basal compartment that is adjacent to the lower surface of the scaffold.

In some embodiments, a method of making a live cell construct for producing milk in culture of the present invention, prior to culturing immortalized mammary epithelial cells, may further comprise: (i) isolating primary mammary epithelial cells, myoepithelial cells, and/or mammary progenitor cells from mammary explants from mammary tissue (e.g., breast, udder, and/or teat tissue), to produce isolated mammary epithelial cells, myoepithelial cells, and/or mammary progenitor cells; (ii) culturing the isolated primary mammary epithelial cells, myoepithelial cells, and/or mammary progenitor cells to produce a mixed population of primary mammary epithelial cells, mammary myoepithelial cells and mammary progenitor cells; (iii) sorting the mixed population of primary mammary epithelial cells, myoepithelial cells, and/or mammary progenitor cells (e.g., selecting the primary mammary epithelial cells) to produce a population of primary mammary epithelial cells; and (iv) stably

introducing (e.g., transfecting/transducing) one or more cells of the population of primary mammary epithelial cells of (iii) with (1) one or more nucleic acids encoding human telomerase reverse transcriptase (hTERT) or simian virus 40 (SV40), or with (2) a small hairpin RNA (shRNA) to p16 (Inhibitor of Cyclin-Dependent Kinase 4) (p16(INK4)) and Master Regulator of Cell Cycle Entry and Proliferative Metabolism (c-MYC) to produce immortalized mammary epithelial cells. In some embodiments, the immortalized cell line may be stably introduced (e.g., transfected/transduced) with (1) one or more nucleic acids encoding hTERT or SV40, and/or (2) a small hairpin RNA (shRNA) to p16 (Inhibitor of Cyclin-Dependent Kinase 4) (p16(INK4)) and Master Regulator of Cell Cycle Entry and Proliferative Metabolism (c-MYC).

In some embodiments, a method of making a live cell construct for producing milk in culture may further comprise storing cells or populations of cells of the present invention (e.g., the live primary mammary epithelial cells, the mixed population primary mammary epithelial cells, myoepithelial cells and mammary progenitor cells, and/or the immortalized mammary epithelial cells) prior to cultivating on a scaffold, optionally wherein the storing is in a freezer or in liquid nitrogen. Storage temperature may depend on the desired storage length. For example, freezer temperature (e.g., storage at a temperature of about 0°C to about -80°C or less, e.g., about 0°C, -10°C, -20°C, -30°C, -40°C, -50°C, -60°C, -70°C, -80°C, -90°C, -100°C or any value or range therein) may be used if the cells are to be used within 6 months (e.g., within 1, 2, 3, 4, 5, or 6 months). For example, liquid nitrogen may be used (e.g., storage at a temperature of -100°C or less (e.g., about -100°C, -110°C, -120°C, -130, -140, -150, -160, -170, -180, -190°C, -200°C, or less) for longer term storage (e.g., storage of 6 months or longer, e.g., 6, 7, 8, 9, 10, 11, or 12 months, or 1, 2, 3, 4, 5, 6 or more years).

In some embodiments, a method of making a live cell construct for producing milk in culture may comprise wherein the isolating and sorting is via fluorescence-activated cell sorting, magnetic-activated cell sorting, and/or microfluidic cell sorting.

In some embodiments, the present invention provides a method of producing milk in culture comprising, culturing a live cell construct comprising (a) a scaffold comprising an upper surface and a lower surface and a continuous (i.e., confluent) polarized monolayer of live primary mammary epithelial cells, a continuous polarized monolayer of a mixed population of live primary mammary epithelial cells, mammary myoepithelial cells and mammary progenitor cells, and/or a continuous polarized monolayer of live immortalized mammary epithelial cells having an apical surface and a basal surface, wherein the

continuous polarized monolayer of live primary mammary epithelial cells, the continuous polarized monolayer of the mixed population of live primary mammary epithelial cells, mammary myoepithelial cells and mammary progenitor cells and/or the continuous polarized monolayer of live immortalized mammary epithelial cells are located on the upper surface of scaffold, (b) a basal compartment and an apical compartment, wherein the lower surface of the scaffold is adjacent to the basal compartment and the apical surface of the continuous polarized monolayer of live primary mammary epithelial cells, the continuous polarized monolayer of the mixed population of live primary mammary epithelial cells, mammary myoepithelial cells and mammary progenitor cells, and/or the continuous polarized monolayer of live immortalized mammary epithelial cells is adjacent to the apical compartment, wherein the continuous polarized monolayer of live primary epithelial mammary cells, the live primary epithelial mammary cells of the continuous polarized monolayer of the mixed population of live primary mammary epithelial cells, mammary myoepithelial cells and mammary progenitor cells, or the continuous polarized monolayer of immortalized mammary epithelial cells excretes milk through its apical surface into the apical compartment, thereby producing milk in culture.

In some embodiments, the monolayer of the live cell construct for the methods of producing milk in culture may be adjacent to the upper surface of the scaffold.

In some embodiments, the live cell construct for the methods of producing milk in culture may further comprise an apical compartment that is adjacent to the apical surface of the monolayer.

In some embodiments, the live cell construct for the methods of producing milk in culture may comprise a basal compartment that is adjacent to the lower surface of the scaffold.

In some embodiments, a method of producing milk in culture of the present invention may further comprise a basal compartment comprising a basal culture medium and the basal culture medium may be in contact with the basal surface of the continuous polarized monolayer of primary mammary epithelial cells, with the basal surface of the continuous polarized the monolayer of the mixed population, or with the basal surface of the continuous polarized monolayer of live immortalized mammary epithelial cells. The basal culture medium may comprise a carbon source, a chemical buffering system, one or more essential amino acids, one or more vitamins and/or cofactors, and one or more inorganic salts.

In some embodiments, the basal culture medium may comprise a carbon source in an amount from about 1 g/L to about 15 g/L of basal culture medium (e.g., about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14 or 15 g/L or any value or range therein), or about 1, 2, 3, 4, 5 or 6 g/L to about 7, 8, 9, or 10, 11, 12, 13, 14 or 15 g/L of the basal culture medium. In some embodiments, the carbon source may include, but is not limited to, be glucose and/or pyruvate. For example, in some embodiments, the basal culture medium may comprise glucose in an amount from about 1g/L to about 12 g/L of basal culture medium, e.g., about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, or 12 g/L or any value or range therein. In some embodiments, the basal culture medium may comprise glucose in an amount from about 1 g/L to about 6 g/L, about 4g/L to about 12 g/L, about 2.5 g/L to about 10.5 g/L, about 1.5g/L to about 11.5 g/L, or about 2g/L to about 10 g/L of basal culture medium. In some embodiments, the basal culture medium may comprise pyruvate at an amount of about 5 g/L to about 15 g/L of basal culture medium, e.g., about 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, or 15 g/L or any value or range therein. In some embodiments, the basal culture medium may comprise pyruvate in an amount from about 5 g/L to about 14.5 g/L, about 10g/L to about 15 g/L, about 7.5 g/L to about 10.5 g/L, about 5.5 g/L to about 14.5 g/L, or about 8g/L to about 10 g/L of basal culture medium.

In some embodiments, the basal culture medium may comprise a chemical buffering system in an amount from about 1g/L to about 4 g/L (e.g., about 1, 1.5, 2, 2.5, 3, 3.5, or 4 g/L or any value or range therein) of basal culture medium or about 10 mM to about 25 mM (e.g., about 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, or 25 mM or any value or range therein). In some embodiments, the chemical buffering system may include, but is not limited to, sodium bicarbonate and/or HEPES. For example, in some embodiments, the basal culture medium may comprise sodium bicarbonate in an amount from about 1g/L to about 4 g/L of basal culture medium, e.g., about 1, 1.5, 2, 2.5, 3, 3.5, or 4 g/L or any value or range therein. In some embodiments, the basal culture medium may comprise sodium bicarbonate in an amount from about 1 g/L to about 3.75 g/L, about 1.25 g/L to about 4 g/L, about 2.5 g/L to about 3 g/L, about 1.5g/L to about 4 g/L, or about 2g/L to about 3.5 g/L of basal culture medium. In some embodiments, the basal culture medium may comprise HEPES in an amount from about 10 mM to about 25 mM, e.g., about 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, or 25 mM or any value or range therein. In some embodiments, the basal culture medium may comprise HEPES in an amount from about 11mM to about 25 mM,

about 10 mM to about 20 mM, about 12.5 mM to about 22.5 mM, about 15 mM to about 20.75 mM, or about 10 mM to about 20 mM.

In some embodiments, the basal culture medium may comprise one or more essential amino acids in an amount from about 0.5 mM to about 5 mM (e.g., about 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5, or 5 mM or any value or range therein) or about 0.5, 1, 1.5, 2 mM to about 2.5, 3, 3.5, 4, 4.5, or 5 mM. In some embodiments, exemplary one or more essential amino acids may be arginine and/or cysteine. For example, in some embodiments, the basal culture medium may comprise arginine in an amount from about 0.5 mM to about 5 mM, e.g., about 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5, or 5 mM or any value or range therein. In some embodiments, the basal culture medium may comprise arginine in an amount from about 0.5 mM to about 4.75 mM, about 2 mM to about 3.5 mM, about 0.5 mM to about 3.5 mM, about 1 mM to about 5 mM, or about 3.5 mM to about 5 mM. For example, in some embodiments, the basal culture medium may comprise cysteine in an amount from about 0.5 mM to about 5 mM, e.g., about 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5, or 5 mM or any value or range therein. In some embodiments, the basal culture medium may comprise cysteine in an amount from about 0.5 mM to about 4.75 mM, about 2 mM to about 3.5 mM, about 0.5 mM to about 3.5 mM, about 1 mM to about 5 mM, or about 3.5 mM to about 5 mM.

In some embodiments, the basal culture medium may comprise one or more vitamins and/or cofactors in an amount from about 0.01 µM to about 50 µM (e.g., about 0.01, 0.02, 0.03, 0.04, 0.05, 0.06, 0.07, 0.08, 0.09, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1, 1.1, 1.2, 1.3, 1.4, 1.5, 1.6, 1.7, 1.8, 1.9, 2, 2.1, 2.2, 2.3, 2.4, 2.5, 3, 4, 5, 6, 7, 8, 9, 10, 12.5, 15, 17.5, 20, 25, 30, 35, 40, 45, 46, 47, 48, 49, 49.025, 49.05, 49.075, or 50 µM or any value or range therein) or about 0.01, 0.02, 0.03, 0.04, 0.05, 0.06, 0.07, 0.08, 0.09, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, or 0.9 µM to about 1, 1.1, 1.2, 1.3, 1.4, 1.5, 1.6, 1.7, 1.8, 1.9, 2, 2.1, 2.2, 2.3, 2.4, 2.5, 3, 4, 5, 6 µM or about 0.02, 0.025, 0.05, 0.075, 1, 1.5, 2, 3, 4, 5, 6, 7, 8, 9, 10 µM to about 12.5, 15, 17.5, 20, 25, 30, 35, 40, 45, 46, 47, 48, 49, 49.025, 49.05, 49.075, or 50 µM. In some embodiments, one or more vitamins and/or cofactors may include, but is not limited to, thiamine and/or riboflavin. For example, in some embodiments, the basal culture medium may comprise thiamine in an amount from about 0.025 µM to about 50 µM, e.g., 0.025, 0.05, 0.075, 1, 1.5, 2, 3, 4, 5, 6, 7, 8, 9, 10, 12.5, 15, 17.5, 20, 25, 30, 35, 40, 45, 46, 47, 48, 49, 49.025, 49.05, 49.075, or 50 µM or any value or range therein. In some embodiments, the basal culture medium may comprise thiamine in an amount from about 0.025 µM to about 45.075 µM, about 1 µM to about 40 µM, about 5 µM to about 35.075 µM, about 10 µM to

about 50 μM, or about 0.05 μM to about 45.5 μM. In some embodiments, the basal culture medium may comprise riboflavin in an amount from about 0.01 μM to about 3 μM, e.g., 0.01, 0.02, 0.03, 0.04, 0.05, 0.06, 0.07, 0.08, 0.09, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1, 1.1, 1.2, 1.3, 1.4, 1.5, 1.6, 1.7, 1.8, 1.9, 2, 2.1, 2.2, 2.3, 2.4, 2.5, 2.6, 2.7, 2.8, 2.9, or 3 μM or any value or range therein. In some embodiments, the basal culture medium may comprise riboflavin in an amount from about 0.01 μM to about 2.05 μM, about 1 μM to about 2.95 μM, about 0.05 μM to about 3 μM, about 0.08 μM to about 1.55 μM, or about 0.05 μM to about 2.9 μM.

In some embodiments, the basal culture medium may comprise one or more inorganic salts in an amount from about 100 mg/L to about 150 mg/L of basal culture medium (e.g., about 100, 105, 110, 115, 120, 125, 130, 135, 140, 145, or 150 mg/L or any value or range therein) or about 100 mg/L to about 150 mg/L of basal culture medium (e.g., about 100, 105, 110, 115, 120, 125, 130, 135, 140, 145, or 150 mg/L or any value or range therein). In some embodiments, exemplary one or more inorganic salts may be calcium and/or magnesium. For example, in some embodiments, the basal culture medium may comprise calcium in an amount from about 100 mg/L to about 150 mg/L of basal culture medium, e.g., about 100, 105, 110, 115, 120, 125, 130, 135, 140, 145, or 150 mg/L or any value or range therein. In some embodiments, the basal culture medium may comprise arginine in an amount from about 100 mg/L to about 125 mg/L, about 105 mg/L to about 150 mg/L, about 120 mg/L to about 130 mg/L, or about 100 mg/L to about 145 mg/L of basal culture medium. In some embodiments, the basal culture medium may comprise magnesium in an amount from f about 0.01 mM to about 1 mM, e.g., about 0.01, 0.02, 0.03, 0.04, 0.05, 0.06, 0.07, 0.08, 0.09, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 0.91, 0.92, 0.93, 0.94, 0.95, 0.96, 0.97, 0.98, 0.99, or 1 mM or any value or range therein. In some embodiments, the basal culture medium may comprise magnesium in an amount from about 0.05 mM to about 1 mM, about 0.01 mM to about 0.78 mM, about 0.5 mM to about 1 mM, about 0.03 mM to about 0.75 mM, or about 0.25 mM to about 0.95 mM.

In some embodiments, the carbon source, chemical buffering system, one or more essential amino acids, one or more vitamins and/or cofactors, and/or one or more inorganic salts may be food grade.

In some embodiments, the basal culture medium may be lactogenic culture medium, e.g., the basal culture medium may further comprise prolactin (e.g., mammalian prolactin, e.g., human prolactin). For example, in some embodiments, the basal culture medium may

comprise prolactin (or prolactin may be added) in an amount from about 20 ng/mL to about 200 ng/L of basal culture medium, e.g., about 20, 30, 40, 50, 60, 70, 80, 90, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190, or 200 ng/mL or any value or range therein. In some embodiments, the basal culture medium may comprise prolactin (or prolactin may be added) in an amount from about 20 ng/mL to about 195 ng/mL, about 50 ng/mL to about 150 ng/mL, about 25 ng/mL to about 175 ng/mL, about 45 ng/mL to about 200 ng/mL, or about 75 ng/mL to about 190 ng/mL of basal culture medium. In some embodiments, the methods of the present invention may further comprise adding prolactin to the basal culture medium, thereby providing a lactogenic culture medium. In some embodiments, the prolactin may be produced by a microbial cell and/or a human cell expressing a recombinant prolactin (e.g., a prolactin comprising a substitution of a serine residue at position 179 of the prolactin gene with aspartate (S179D), e.g., S179D-prolactin). In some embodiments, adding prolactin to the basal culture medium may comprise conditioning basal culture medium by culturing cells that express and secrete prolactin, and applying the conditioned basal culture medium comprising prolactin to the basal surface of the monolayer of primary mammary epithelial cells, the basal surface of the monolayer of the mixed population, or the basal surface of the monolayer of live immortalized mammary epithelial cells.

In some embodiments, the basal culture medium may further comprise other factors to improve efficiency, including, but not limited to, insulin, an epidermal growth factor, and/or a hydrocortisone. In some embodiments, the methods of the present invention may further comprise adding other factors (e.g., insulin, an epidermal growth factor, and/or a hydrocortisone) to the basal culture medium, e.g., to improve efficiency.

In some embodiments, the methods of the present invention may comprise monitoring the glucose concentration and/or rate of glucose consumption in the basal culture medium and/or in the lactogenic culture medium. In some embodiments, the prolactin may be added when the rate of glucose consumption in the basal culture medium is steady state.

In some embodiments, a method of producing milk in culture may comprise culturing at a temperature of about 35°C to about 39°C (e.g., a temperature of about 35°C, 35.5°C, 36°C, 36.5°C, 37°C, 37.5°C, 38°C, 38.5°C or about 39°C, or any value or range therein, e.g., about 35°C to about 38°C, about 36% to about 39°C, about 36.5°C to about 39°C, about 36.5°C to about 38°C, or about 36.5°C to about 37.5°C). In some embodiments, the culturing may be carried out at a temperature of about 37°C.

In some embodiments, a method of producing milk in culture may comprise culturing at an atmospheric concentration of CO_2 of about 4% to about 6%, e.g., an atmospheric concentration of CO_2 of about 4%, 4.25%, 4.5%, 4.75%, 5%, 5.25%, 5.5%, 5.75%, or 6% or any value or range therein, e.g., about 4% to about 5.5%, about 4.5% to about 6%, about 4.5% to about 5.5%, or about 5% to about 6%). In some embodiments, the culturing may be carried out at an atmospheric concentration of CO_2 of about 5%.

In some embodiments, a method of producing milk in culture may comprise monitoring the concentration of dissolved O_2 and CO_2. In some embodiments, the concentration of dissolved O_2 may be maintained between about 10% to about 25% or any value or range therein (e.g., about 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, or 25%). For example, in some embodiments, the concentration of dissolved O_2 may be maintained between about 12% to about 25%, about 15% to about 22%, about 10% to about 20%, about 15%, about 20%, or about 22%. In some embodiments, the concentration of CO_2 may be maintained between about 4% to about 6%, e.g., a concentration of CO_2 of about 4%, 4.25%, 4.5%, 4.75%, 5%, 5.25%, 5.5%, 5.75%, or 6% or any value or range therein, e.g., about 4% to about 5.5%, about 4.5% to about 6%, about 4.5% to about 5.5%, or about 5% to about 6%). In some embodiments, the concentration of CO_2 may be maintained at about 5%.

In some embodiments, a method of producing milk in culture may further comprise applying a transepithelial electrical resistance (TEER) to measure the maintenance of the monolayer of epithelial cells. TEER measures a voltage difference between the fluids (e.g., media) in two compartments (e.g., between the apical and basal compartments), wherein if the barrier between the compartments loses integrity, the fluids in the two compartments may mix. When there is fluid mixing, there will be no voltage difference; a voltage difference indicates that the barrier is intact. Upon detection of a loss of voltage by TEER, a scaffold (e.g., a transwell filter, a microstructured bioreactor, a decellularized tissue, a hollow fiber bioreactor, etc.) may be reinoculated with additional cells and allowed time to reestablish a barrier (e.g., a confluent, continuous monolayer) before resuming methods of the present invention (e.g., milk production).

In some embodiments, a method of producing milk in culture may further comprise storing cells or populations of cells of the present invention (e.g., the live primary mammary epithelial cells, the mixed population primary mammary epithelial cells, myoepithelial cells and mammary progenitor cells, and/or the immortalized mammary epithelial cells) prior to cultivating on a scaffold, optionally wherein the storing is in a freezer or in liquid nitrogen.

Storage temperature may depend on the desired storage length. For example, freezer temperature (e.g., storage at a temperature of about 0°C to about -80°C or less, e.g., about 0°C, -10°C, -20°C, -30°C, -40°C, -50°C, -60°C, -70°C, -80°C, -90°C, -100°C or any value or range therein) may be used if the cells are to be used within 6 months (e.g., within 1, 2, 3, 4, 5, or 6 months). For example, liquid nitrogen may be used (e.g., storage at a temperature of -100°C or less (e.g., about -100°C, -110°C, -120°C, -130, -140, -150, -160, -170, -180, -190°C, -200°C, or less) for longer term storage (e.g., storage of 6 months or longer, e.g., 6, 7, 8, 9, 10, 11, or 12 months, or 1, 2, 3, 4, 5, 6 or more years).

In some embodiments, a method of producing milk in culture may further comprise comprising collecting the milk from the apical compartment to produce collected milk. In some embodiments, the collecting may be via a port, via gravity, and/or via a vacuum. In some embodiments, a vacuum may be attached to a port.

In some embodiments, a method of producing milk in culture may further comprise freezing the collected milk to produce frozen milk and/or lyophilizing the collected milk to produce lyophilized milk.

In some embodiments, a method of producing milk in culture may further comprise packaging the collected milk, the frozen milk and/or the lyophilized milk into a container.

In some embodiments, a method of producing milk in culture may further comprise extracting one or more components from the collected milk. Non-limiting examples of components from the collected milk include milk protein, lipid, carbohydrate, vitamin, and/or mineral contents. In some embodiments, the components from the collected milk may be lyophilized and/or concentrated to produce a lyophilized or a concentrated milk component product. In some embodiments, the components from the collected milk may concentrated by, e.g., membrane filtration and/or reverse osmosis. In some embodiments, the lyophilized or concentrated milk component product may be packaged in a container, optionally wherein the container is sterile and/or a food grade container. In some embodiments, the container may be vacuum-sealed. In some embodiments, the container may be a canister, a jar, a bottle, a bag, a box, or a pouch.

The present invention also provides a method of producing a modified primary mammary epithelial cell or a immortalized mammary epithelial cell, wherein the method comprises introducing into the cell: (a) a polynucleotide encoding a prolactin receptor comprising a modified intracellular signaling domain, optionally wherein the prolactin receptor comprises a truncation wherein position 154 of exon 10 has been spliced to the 3'

sequence of exon 11; (b) a polynucleotide encoding a chimeric prolactin receptor that binds to a ligand, which is capable of activating milk synthesis in the absence of prolactin; (c) a polynucleotide encoding a constitutively or conditionally active prolactin receptor protein, optionally wherein the polynucleotide encodes a constitutively active human prolactin receptor protein comprising a deletion of amino acids 9 through 187 (e.g., a deletion of amino acids 9 through 187, wherein the numbering is based on the reference amino acid sequence of a human prolactin receptor identified as SEQ ID NO:1); (d) a polynucleotide encoding a modified (e.g., recombinant) effector of a prolactin protein comprising (i) a janus kinase-2 (JAK2) tyrosine kinase domain, optionally wherein the JAK2 tyrosine kinase domain may be fused to a signal transducer and activator of transcription-5 (STAT5) tyrosine kinase domain (e.g., a polynucleotide encoding a JAK2 tyrosine kinase domain linked to the 3' end of a polynucleotide encoding the STAT5 tyrosine kinase domain); and/or (ii) a prolactin receptor intracellular domain fused to a JAK2 tyrosine kinase domain; (e) a loss of function mutation into a circadian related gene *PER2* (period circadian protein homolog 2); and/or (f) a polynucleotide encoding one or more glucose transporter genes GLUT1 and/or GLUT12, thereby increasing the rate of nutrient uptake at the basal surface of the monolayer.

In some embodiments, a constitutively active human prolactin receptor protein may comprise a deletion of amino acids 9 through 187, wherein the numbering is based on the reference amino acid sequence of a human prolactin receptor identified as SEQ ID NO:1.

SEQ ID NO:1: Human prolactin receptor (GenBank accession number AAD32032.1)

MKENVASATVFTLLLFLNTCLLNGQLPPGKPEIFKCRSPNKETFTCWWRPGTDGGLPTNYSLTYHREGET
LMHECPDYITGGPNSCHFGKQYTSMWRTYIMMVNATNQMGSSFSDELYVDVTYIVQPDPPLELAVEVKQP
EDRKPYLWIKWSPPTLIDLKTGWFTLLYEIRLKPEKAAEWEIHFAGQQTEFKILSLHPGQKYLVQVRCKP
DHGYWSAWSPATFIQIPSDFTMNDTTVWISVAVLSAVICLIIVWAVALKGYSMVTCIFPPVPGPKIKGFD
AHLLEKGKSEELLSALGCQDFPPTSDYEDLLVEYLEVDDSEDQHLMSVHSKEHPSQGMKPTYLDPDTDSG
RGSCDSPSLLSEKCEEPQANPSTFYDPEVIEKPENPETTHTWDPQCISMEGKIPYFHAGGSKCSTWPLPQ
PSQHNPRSSYHNITDVCELAVGPAGAPATLLNEAGKDALKSSQTIKSREEGKATQQREVESFHSETDQDT
PWLLPQEKTPFGSAKPLDYVEIHKVNKDGALSLLPKQRENSGKPKKPGTPENNKEYAKVSGVMDNNILVL
VPDPHAKNVACFEESAKEAPPSLEQNQAEKALANFTATSSKCRLQLGGLDYLDPACFTHSFH

In some embodiments, a constitutively active human prolactin receptor protein may comprise a deletion of the following amino acids:

VFTLLLFLNTCLLNGQLPPGKPEIFKCRSPNKETFTCWWRPGTDGGLPTNYSLTYHRE
GETLMHECPDYITGGPNSCHFGKQYTSMWRTYIMMVNATNQMGSSFSDELYVDVT
YIVQPDPPLELAVEVKQPEDRKPYLWIKWSPPTLIDLKTGWFTLLYEIRLKPEKAA

(e.g., amino acid positions9 through 187 of SEQ ID NO:1).

In some embodiments, a loss of function mutation introduced into a circadian related gene *PER2* may comprise an 87-amino acid deletion from position 348 to 434 in *PER2*, wherein the numbering is based on the reference amino acid sequence of a human PER2 identified as SEQ ID NO:2.

SEQ ID NO:2: Human Period circadian protein homolog 2 (GenBank accession number NM_022817)

MNGYAEFPPSPSNPTKEPVEPQPSQVPLQEDVDMSSGSSGHETNENCSTGRDSQGSDCDDSGKEL
GMLVEPPDARQSPDTFSLMMAKSEHNPSTSGCSSDQSSKVDTHKELIKTLKELKVHLPADKKAKG
KASTLATLKYALRSVKQVKANEEYYQLLMSSEGHPCGADVPSYTVEEMESVTSEHIVKNADMFA
VAVSLVSGKILYISDQ VASIFHCKRDAFSDAKFVEFLAPHDVGVFHSFTSPYKLPLWSMCSGADSF
TQECMEEKSFFCRVSVRKSHENEIRYHPFRMTPYLVKVRDQQGAESQLCCLLLAERVHSGYEAPR
IPPEKRIFTTTHTPN**CLFQDVDERAVPLLGYLPQDLIETPVLVQLHPSDRPLMLAIHKKILQSG**
GQPFDYSPIRFRARNGEYITLDTSWSSFINPWSRKISFIIGRHKVRVGPLNEDVFAAHPCTEEKA
LHPSIQ ELTEQIHRLL LQPVPHSGSS GYGSLGSNGSHEHLMSQTSSSDSNGHEDSRRRRAEICKNG
NKTKNRSHYSHESGEQKKKSVTEMQTNPPAEKKAVPAMEKDSLGVSFPEELACKNQPTCSYQQIS
CLDSV IRYLESCNEAATLKRKCEFP ANVPALRSSDKRKATVSPGPHAGEAEPPSRVNSRTGVGTH
LTSLALPGKAESVASLTSQCSYSSTIVHVGDKKPQPELEMVEDAASGPESLDCLAGPALACGLSQE
KEPFKKLGLTKEVLAAHTQKEEQSFLQKFKEIRKLSIFQSHCHYYLQERSKGQPSERTAPGLRNTS
GIDSPWKKTGKNRKLKSKRVKPRDSSESTGSGGPVSARPPLVGLNATAWSPSDTSQSSCPAVPFPA
PVPAAYSLPVFPAPGTVAAPPAPPHASFTVPAVPVDLQHQFAVQPPPFPAPLAPVMAFMLPSYSFP
SGTPNLPQAFFPSQPQFPSHPTLTSEMASASQPEFPEGGTGAMGTTGATETAAVGADCKPGTSRDQ
QPKAPLTRDEPSDTQNSDALSTSSGLLNLLLNEDLCSASGSAASESLGSGSLGCDASPSGAGSSDTS
HTSKYFGSIDSSENNHKAKMNTGMEESEHFIKCVLQDPIWLLMADADSSVMMTYQLPSRNLEAV
LKEDREKLKLLQKLQPRFTESQKQELREVHQWMQTGGLPAAIDVAECVYCENKEKGNICIPYEED
IPSLGLSEVSDTKE DENGSPLNHRIEEQT

In some embodiments, a loss of function mutation introduced into a circadian related gene *PER2* may comprise a deletion of the following amino acids: CLFQDVDERAVPLLGYLPQDLIETPVLVQLHPSDRPLMLAIHKKILQSGGQPFDYSPIR FRARNGEYITLDTSWSSFINPWSRKISFIIGRHKV (e.g., amino acid positions 348 through 434 of SEQ ID NO:2).

In some embodiments, a polynucleotide encoding a prolactin receptor comprising a modified intracellular signaling domain, optionally wherein the prolactin receptor comprises a truncation wherein position 154 of exon 10 has been spliced to the 3' sequence of exon 11, may encode the following amino acid sequence identified as SEQ ID NO:3.

SEQ ID NO:3: Human isoform 4 of Prolactin receptor (GenBank accession number AF416619; Trott et al. 2003 *J. Mol. Endocrinol.* 30(1):31-47)

MKENVASATVFTLLLFLNTCLLNGQLPPGKPEIFKCRSPNKETFTCWWRPGTDGGLPTNYSLTYHREGETLMHEC
PDYITGGPNSCHFGKQYTSMWRTYIMMVNATNQMGSSFSDELYVDVTYIVQPDPPLELAVEVKQPEDRKPYLWIK
WSPPTLIDLKTGWFTLLYEIRLKPEKAAEWEIHFAGQQTEFKILSLHPGQKYLVQVRCKPDHGYWSAWSPATFIQ
IPSDFTMNDTTVWISVAVLSAVICLIIVWAVALKGYSMVTCIFPPVPGPKIKGFDAHLLEKGKSEELLSALGCQD
FPPTSDYEDLLVEYLEVDDSEDQHLMSVHSKEHPSQGDPLMLGASHYKNLKSYRPRKISSQGRLAVFTKATLTTV
Q

In some embodiments, a polynucleotide encoding a modified (e.g., recombinant) effector of a prolactin protein comprising (i) a janus kinase-2 (JAK2) tyrosine kinase domain, optionally wherein the JAK2 tyrosine kinase domain may be fused to a signal transducer and activator of transcription-5 (STAT5) tyrosine kinase domain (e.g., a polynucleotide encoding a JAK2 tyrosine kinase domain linked to the 3' end of a polynucleotide encoding the STAT5 tyrosine kinase domain) may encode the following amino acid sequence identified as SEQ ID NO:4. Bolded amino acids correspond to the JAK2 kinase domain of amino acid positions 757 through 1129 of a reference human JAK2 amino acid sequence.

SEQ ID NO:4. STA5A Human signal transducer and activator of transcription 5A fused at 3' end to amino acids 757-1129 of JAK2 human tyrosine-protein kinase

MAGWIQAQQL QGDALRQMQV LYGQHFPIEV RHYLAQWIES QPWDAIDLDN PQDRAQATQL
LEGLVQELQK KAEHQVGEDG FLLKIKLGHY ATQLQKTYDR CPLELVRCIR HILYNEQRLV
REANNCSSPA GILVDAMSQK HLQINQTFEE LRLVTQDTEN ELKKLQQTQE YFIIQYQESL
RIQAQFAQLA QLSPQERLSR ETALQQKQVS LEAWLQREAQ TLQQYRVELA EKHQKTLQLL
RKQQTIILDD ELIQWKRRQQ LAGNGGPPEG SLDVLQSWCE KLAEIIWQNR QQIRRAEHLC
QQLPIPGPVE EMLAEVNATI TDIISALVTS TFIIEKQPPQ VLKTQTKFAA TVRLLVGGKL
NVHMNPPQVK ATIISEQQAK SLLKNENTRN ECSGEILNNC CVMEYHQATG TLSAHFRNMS
LKRIKRADRR GAESVTEEKF TVLFESQFSV GSNELVFQVK TLSLPVVVIV HGSQDHNATA
TVLWDNAFAE PGRVPFAVPD KVLWPQLCEA LNMKFKAEVQ SNRGLTKENL VFLAQKLFNN
SSSHLEDYSG LSVSWSQFNR ENLPGWNYTF WQWFDGVMEV LKKHHKPHWN DGAILGFVNK
QQAHDLLINK PDGTFLLRFS DSEIGGITIA WKFDSPERNL WNLKPFTTRD FSIRSLADRL
GDLSYLIYVF PDRFKDEVFS KYYTPVLAKA VDGYVKPQIK QVVPEFVNAS ADAGGSSATY
MDQAPSPAVC PQAPYNMYPQ NPDHVLDQDG EFDLDETMDV ARHVEELLRR PMDSLDSRLS
PPAGLFTSAR GSLSLDSQ **RKLQFYEDRH QLPAPKWAEL ANLINNCMDY EPDFRPSFRA**
IIRDLNSLFT PDYELLTEND MLPNMRIGAL GFSGAFEDRD PTQFEERHLK FLQQLGKGNF
GSVEMCRYDP LQDNTGEVVA VKKLQHSTEE HLRDFEREIE ILKSLQHDNI VKYKGVCYSA
GRRNLKLIME YLPYGSLRDY LQKHKERIDH IKLLQYTSQI CKGMEYLGTK RYIHRDLATR
NILVENENRV KIGDFGLTKV LPQDKEYYKV KEPGESPIFW YAPESLTESK FSVASDVWSF
GVVLYELFTY IEKSKSPPAE FMRMIGNDKQ GQMIVFHLIE LLKNNGRLPR PDGCPDEIYM
IMTECWNNNV NQRPSFRDLA LRVDQIRDN

Having described the present invention, the same will be explained in greater detail in the following examples, which are included herein for illustration purposes only, and which are not intended to be limiting to the invention.

EXAMPLES

EXAMPLE 1:

A cell culture system designed for the collection of milk should support compartmentalized secretion of the product such that the milk is not exposed to the media that provides nutrients to the cells. In the body, milk-producing epithelial cells line the interior surface of the mammary gland as a continuous monolayer. The monolayer is oriented such that the basal surface is attached to an underlying basement membrane, while milk is secreted from the apical surface and stored in the luminal compartment of the gland, or alveolus, until it is removed during milking or feeding. Tight junctions along the lateral surfaces of the cells ensure a barrier between the underlying tissues and the milk located in the alveolar compartment. Therefore, *in vivo*, the tissue of the mammary gland is arranged such that milk secretion is compartmentalized, with the mammary epithelial cells themselves establishing the interface and maintaining the directional absorption of nutrients and secretion of milk.

The present invention describes a cell culture apparatus that recapitulates the compartmentalizing capability of the mammary gland that may be used to collect milk from mammary epithelial cells grown outside of the body. Such an apparatus can include a scaffold to support the proliferation of mammary cells at the interface between two compartments, such that the epithelial monolayer provides a physical boundary between the nutrient medium and the secreted milk. In addition to providing a surface for growth, the scaffold provides spatial cues that guide the polarization of the cells and ensures the directionality of absorption and secretion. This invention describes the preparation, cultivation, and stimulation of mammary epithelial cells in a compartmentalizing cell culture apparatus for the production and collection of milk for nutritional use (*see* e.g., **FIG. 1**).

Preparation of mammary epithelial cells. Mammary epithelial cells are obtained from surgical explants of dissected mammary tissue (e.g., breast, udder, teat). Generally, after surgical dissection of the mammary tissue, any fatty or stromal tissue is manually removed under aseptic conditions, and the remaining tissue of the mammary gland is enzymatically digested with collagenase and/or hyaluronidase prepared in a chemically defined nutrient media, which should be composed of ingredients that are "generally recognized as safe" (GRAS). The sample is maintained at 37 °C with gentle agitation. After digestion, a suspension of single cells or organoids is collected, either by centrifugation or by pouring the sample through a sterile nylon cell strainer. The cell suspension is then transferred to a tissue

culture plate coated with appropriate extracellular matrix components (e.g., collagen, laminin, fibronectin).

Alternatively, explant specimens can be processed into small pieces, for example by mincing with a sterile scalpel. The tissue pieces are plated onto a suitable surface such as a gelatin sponge or a plastic tissue culture plate coated with appropriate extracellular matrix.

The plated cells are maintained at 37 °C in a humidified incubator with an atmosphere of 5% CO_2. During incubation, the media is exchanged about every 1 to 3 days and the cells are sub-cultured until a sufficient viable cell number is achieved for subsequent processing, which may include preparation for storage in liquid nitrogen; development of immortalized cell lines through the stable transfection of genes such as SV40, TERT, or other genes associated with senescence; isolation of mammary epithelial, myoepithelial, and stem/progenitor cell types by, for example, fluorescence-activated cell sorting; and/or introduction into a compartmentalizing tissue culture apparatus for the production and collection of milk for human consumption.

Cultivation of mammary epithelial cells for the production of milk. Milk for nutritional use is produced by mammary epithelial cells isolated as described above and cultured in a format that supports compartmentalized secretion such that separation between the nutrient medium and the product is maintained. The system relies on the ability of mammary epithelial cells to establish a continuous monolayer with appropriate apical-basal polarity when seeded onto an appropriate scaffold positioned at the interface between the apical compartment, into which milk is secreted, and the basal compartment, through which nutrient media is provided (*see*, e.g., **FIG. 2**). Transwell filters placed in tissue culture plates, as well as bioreactors based on hollow fiber or microstructured scaffolds, for example, may be used to support these characteristics.

Following the isolation and expansion of mammary epithelial cells, the cells are suspended in a chemically defined nutrient medium composed of food-grade components and inoculated into a culture apparatus that has been pre-coated with a mixture of extracellular matrix proteins, such as collagen, laminin, and/or fibronectin. The cell culture apparatus may be any design that allows for the compartmentalized absorption of nutrients and secretion of product from a polarized, confluent, epithelial monolayer. Examples include hollow fiber and microstructured scaffold bioreactors (*see*, e.g., **FIGS. 3** and **4**, respectively). Alternatives include other methods of 3-dimensional tissue culture, such as the preparation of decellularized mammary gland as a scaffold, repopulated with stem cells to produce a

functional organ *in vitro*, or collection of milk from the lumen of mammary epithelial cell organoids or "mammospheres" grown either in a hydrogel matrix or in suspension.

The apparatus includes sealed housing that maintains a temperature of about 37 °C in a humidified atmosphere of about 5% CO_2. Glucose uptake is monitored to evaluate the growth of the culture as the cells proliferate within the bioreactor. Stabilization of glucose consumption indicates that the cells have reached a confluent, contact-inhibited state. The integrity of the monolayer is ensured using transepithelial electrical resistance. Sensors monitor concentrations of dissolved O_2 and CO_2 in the media at multiple locations. A computerized pump circulates media through the bioreactor at a rate that balances the delivery of nutrients with the removal of metabolic waste such as ammonia and lactate. Media can be recycled through the system after removal of waste using Lactate Supplementation and Adaptation technology (Freund et al. 2018 *Int J Mol Sci*. 19(2)) or by passing through a chamber of packed zeolite.

Stimulation of milk production. *In vivo* and in cultured mammary epithelial cells, the production and secretion of milk is stimulated by prolactin. In culture, prolactin can be supplied exogenously in the nutrient media at concentrations approximating those observed in the body during lactation, e.g., about 20 ng/ml to about 200 ng/mL. Purified prolactin can be obtained commercially; however, alternative methods of providing prolactin or stimulating lactation may be employed, including expression and purification of the recombinant protein from microbial or mammalian cell cultures. Alternatively, conditioned media prepared by culturing cells that express and secrete prolactin can be applied to mammary epithelial cell cultures to stimulate lactation. Bioreactors can be set up in series such that media passing through a culture of cells expressing prolactin or other key media supplements is conditioned prior to exposure to mammary cells grown in a compartmentalizing culture apparatus as described.

Other approaches to upregulate milk production and/or spare the use of exogenous prolactin include molecular manipulation of the signaling pathways that are regulated by binding of prolactin to its receptor on the surface of mammary epithelial cells, such as the following: (a) expression of constructs targeting the posttranslational modification of prolactin; (b) expression of alternative isotypes of the prolactin receptor; (c) expression of a chimeric prolactin receptor in which the extracellular domain is exchanged with the binding site for a different ligand; (d) introduction of a gene encoding a constitutively or conditionally active prolactin receptor or modified versions of its downstream effectors such as STAT5 or

Akt; (e) knockout or modification of the PER2 circadian gene; and/or (f) molecular approaches aimed at increasing the rate of nutrient uptake at the basal surface of the mammary epithelial monolayer.

Collection of milk. Secreted milk is collected continuously or at intervals through, for example, a port installed in the apical compartment of the culture apparatus. A vacuum may be applied to the port to facilitate collection and may also contribute to the stimulation of further production. The collected milk may be packaged into sterile containers and sealed for distribution, frozen or lyophilized for storage, or processed for the extraction of specific components.

The present invention provides mammary epithelial cell cultures for the production of milk for nutritional use. In addition to human breast milk, this method may be used to produce milk from other mammalian species, for example, for human consumption or veterinary use. Because it has not been previously possible to produce milk outside the body, this technology may result in novel commercial opportunities, in addition to providing an alternative mode of production for existing products. The social and economic effects of the commercial development of this technology are broad and far reaching. Production of human breast milk from cultured cells may provide a means to address infant malnutrition in food-scarce communities, provide essential nutrients to premature infants who are unable to breastfeed, and offer mothers a new option for feeding their babies that provides optimal nutrition with the convenience of infant formula. Production of cow or goat milk provides an opportunity to reduce the environmental, social, and animal welfare effects of animal agriculture. The process described here addresses an important gap in the emerging field of cellular agriculture and introduces an opportunity to dramatically update the human food supply without compromising our biological and cultural attachment to the most fundamental of our nutrition sources.

The foregoing examples are illustrative of the present invention, and are not to be construed as limiting thereof. Although the invention has been described in detail with reference to preferred embodiments, variations and modifications exist within the scope and spirit of the invention as described and defined in the following claims.

FIG. 1

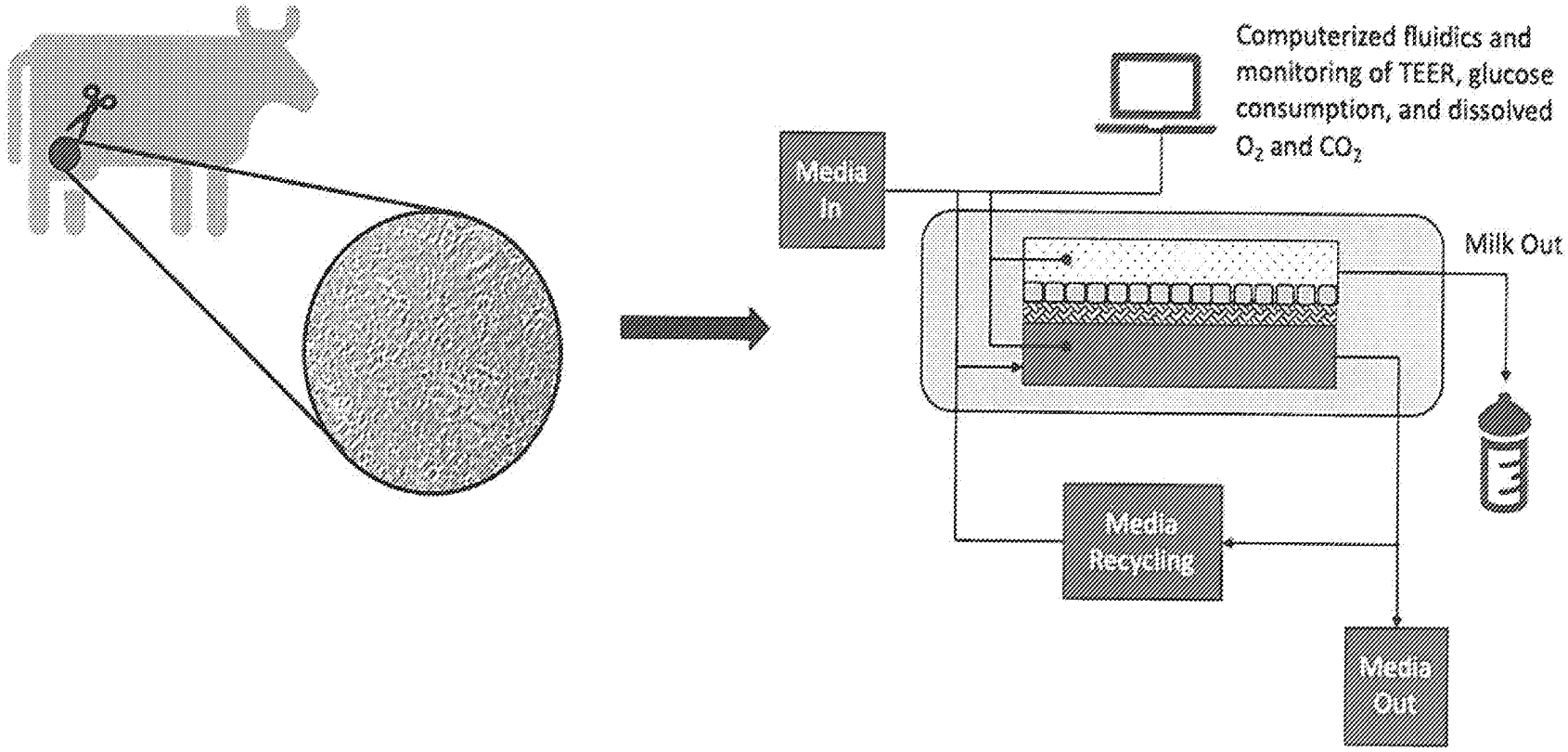


FIG. 2

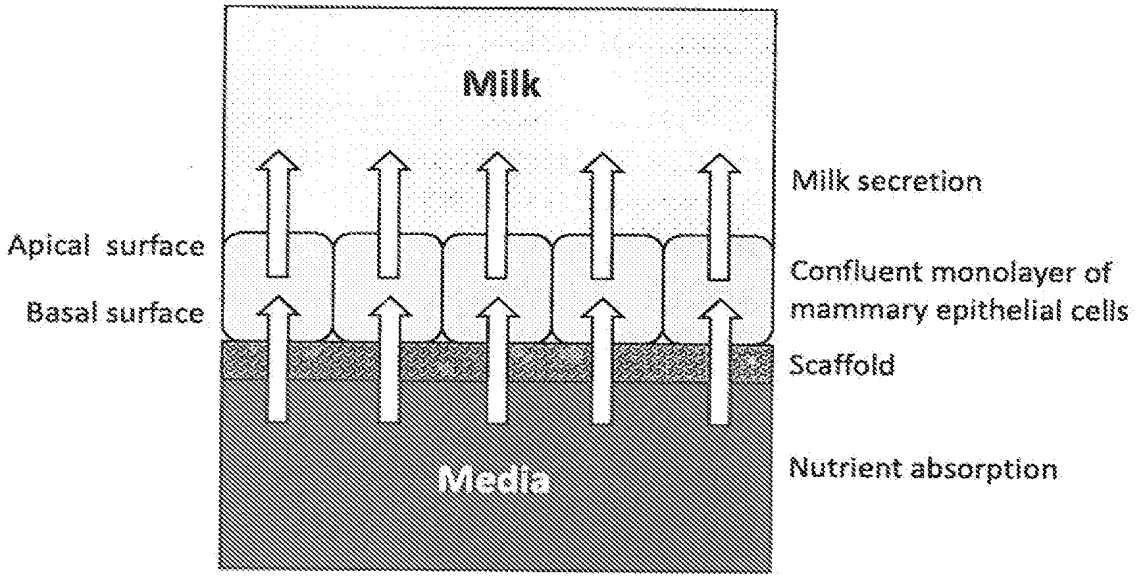

Milk
Milk secretion
Apical surface
Basal surface
Confluent monolayer of mammary epithelial cells
Scaffold
Media
Nutrient absorption

FIG. 3

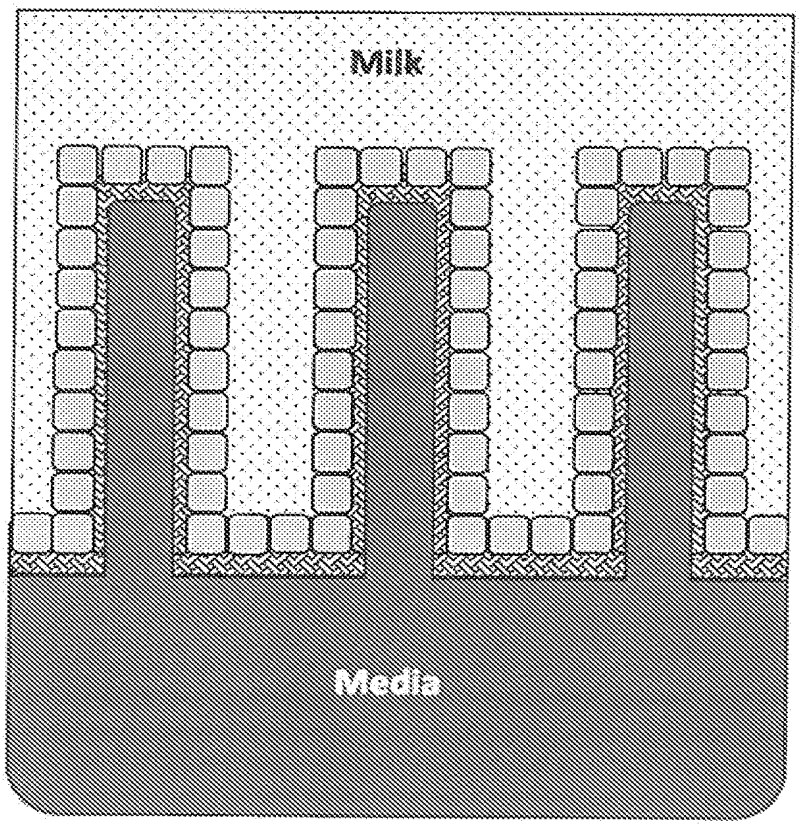
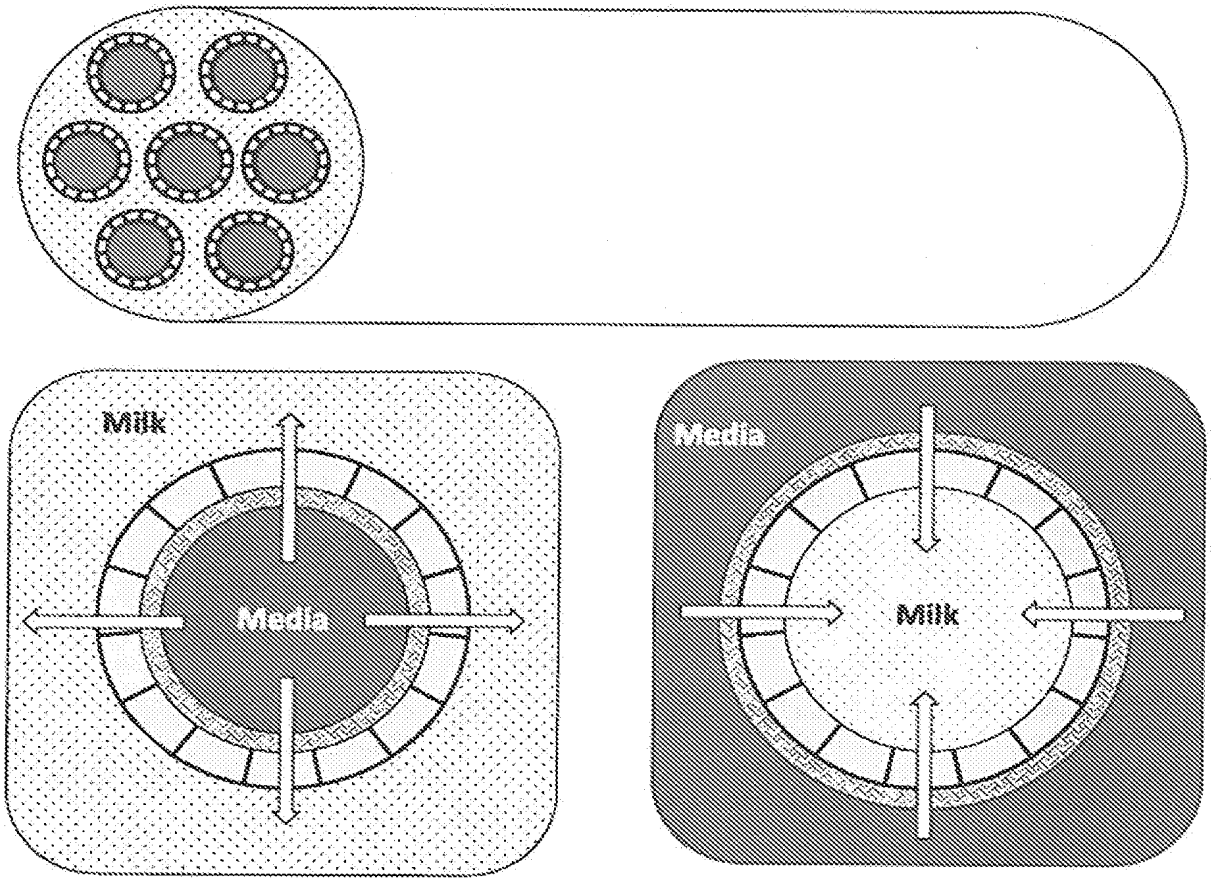

Milk
Media

FIG. 4



What is claimed is:

1. A live cell construct comprising,

a scaffold having a top surface and a bottom surface; and

a continuous monolayer of (a) live primary mammary epithelial cells, (b) a mixed population of live primary mammary epithelial cells, mammary myoepithelial cells and mammary progenitor cells, and/or (c) live immortalized mammary epithelial cells on the top surface of the scaffold, the continuous monolayer of (a) live primary mammary epithelial cells, (b) mixed population of live primary mammary epithelial cells mammary myoepithelial cells and mammary progenitor cells, and/or (c) immortalized mammary epithelial cells having an apical surface and a basal surface (e.g., the cells form a polarized and confluent cell monolayer), wherein the construct comprises an apical compartment above and adjacent to the apical surface of the continuous monolayer of the (a) live primary mammary epithelial cells, the (b) mixed population of live primary mammary epithelial cells, mammary myoepithelial cell and mammary progenitor cells, and/or the (c) immortalized mammary epithelial cells and a basal compartment below and adjacent to the bottom surface of the scaffold.

2. The live cell construct of claim 1, wherein milk produced by the primary mammary epithelial cells or immortalized mammary epithelial cells is excreted through the apical surface of the cells into the apical compartment.

3. The live cell construct of claim 1 or claim 2, wherein the basal compartment comprises a basal culture medium and the basal culture medium is in contact with the basal surface of the live primary mammary epithelial cells, the mixed population of live primary mammary epithelial cells, mammary myoepithelial cells and mammary progenitor cells, and/or the immortalized mammary epithelial cells.

4. The live cell construct of claim 3, wherein the basal culture medium comprises a carbon source, a chemical buffering system, one or more essential amino acids, one or more vitamins and/or cofactors, and one or more inorganic salts.

5. The live cell construct of claim 3 or claim 4, wherein the basal culture medium is a lactogenic culture medium and further comprises prolactin.

6. The live cell construct of any one of claims 1 to 5, wherein the scaffold is fabricated as a 2-dimensional surface (e.g., a transwell filter), a 3-dimensional micropatterned surface (e.g., microstructured bioreactor, decellularized tissue), or as a cylindrical structure that can be assembled into bundles (e.g., hollow fiber bioreactor).

7. The live cell construct of any one of claims 1 to 6, wherein the top surface of the scaffold is coated with one or more extracellular matrix proteins.

8. The live cell construct of claim 6, wherein the one or more extracellular matrix proteins are collagen, laminin, entactin, tenascin, and/or fibronectin.

9. The live cell construct of any one of claims 1 to 8, wherein the scaffold comprises a natural polymer, a biocompatible synthetic polymer, a synthetic peptide, and/or a composite derived from any combination thereof.

10. The live cell construct of claim 9, wherein the natural polymer is collagen, chitosan, cellulose, agarose, alginate, gelatin, elastin, heparan sulfate, chondroitin sulfate, keratan sulfate, and/or hyaluronic acid.

11. The live cell construct of claim 9 or claim 10, wherein the biocompatible synthetic polymer may be polysulfone, polyvinylidene fluoride, polyethylene co-vinyl acetate, polyvinyl alcohol, sodium polyacrylate, an acrylate polymer, and/or polyethylene glycol.

12. The live cell construct of any one of claims 1 to 9, wherein said scaffold is porous.

13. The live cell construct of any one of claims 1 to 13, wherein the live primary mammary epithelial cells, the mixed population of live primary mammary epithelial cells, mammary myoepithelial cells and mammary progenitor cells, and/or the immortalized mammary epithelial cells are from a mammal.

14. The live cell construct of any one of claims 1 to 13, wherein the mammal is a primate (e.g., chimpanzee, orangutan, gorilla, monkey (e.g., Old World, New World), lemur, human), a dog, a cat, a rabbit, a mouse, a rat, a horse, a cow, a goat, a sheep, an ox, a pig, a deer, a musk deer, a bovid, a whale, a dolphin, a hippopotamus, an elephant, a rhinoceros, a giraffe, a zebra, a lion, a cheetah, a tiger, a panda, a red panda, and an otter.

15. The live cell construct of any one of claims 1 to 13, wherein the mammal is from an endangered species.

16. A method of producing milk in culture, the method comprising culturing the live cell construct of any one of claims 1 to 15, thereby producing milk in culture.

17. A method of making a live cell construct for producing milk in culture, the method comprising

(a) isolating primary mammary epithelial cells, myoepithelial cells and/or mammary progenitor cells from mammary explants from mammary tissue, to produce isolated mammary epithelial cells, myoepithelial cells and mammary progenitor cells;

(b) culturing the isolated primary mammary epithelial cells, myoepithelial cells and mammary progenitor cells to produce a mixed population of primary mammary epithelial cells, mammary myoepithelial cells and mammary progenitor cells;

(c) cultivating the mixed population of (b) on a scaffold, the scaffold having an upper surface and lower surface, to produce a polarized, continuous (i.e., confluent) monolayer of primary mammary epithelial cells, myoepithelial cells and mammary progenitor cells of the mixed population on the upper surface of the scaffold, wherein the polarized, continuous monolayer comprises an apical surface and a basal surface, thereby producing a live cell construct for producing milk in culture.

18. The method of claim 17, further comprising storing the isolated primary mammary epithelial cells, myoepithelial cells, and/or mammary progenitor cells of (b) prior to cultivating on a scaffold, optionally wherein the storing is in a freezer or in liquid nitrogen.

19. A method of making a live cell construct for producing milk in culture, the method comprising:

a) isolating primary mammary epithelial cells, myoepithelial cells, and/or mammary progenitor cells from mammary explants from mammary tissue (e.g., breast, udder, teat tissue), to produce isolated mammary epithelial cells, myoepithelial cells, and/or mammary progenitor cells;

(b) culturing the isolated primary mammary epithelial cells, myoepithelial cells, and/or mammary progenitor cells to produce a mixed population of primary mammary epithelial cells, mammary myoepithelial cells and mammary progenitor cells;

(c) sorting the mixed population of primary mammary epithelial cells, myoepithelial cells, and/or mammary progenitor cells to produce a population of primary mammary epithelial cells; and

(d) cultivating the population of primary mammary epithelial on a scaffold, the scaffold having an upper surface and lower surface, to produce a polarized, continuous (i.e., confluent) monolayer of primary mammary epithelial cells on the upper surface of the scaffold, wherein the polarized, continuous monolayer comprises an apical surface and a basal surface, thereby producing a live cell construct for producing milk in culture

20. A method of making a live cell construct for producing milk in culture, the method comprising

(a) culturing immortalized mammary epithelial cells to produce increased numbers of immortalized mammary epithelial cells;

(b) cultivating the immortalized mammary epithelial cells of (a) on a scaffold, the scaffold having an upper surface and lower surface, to produce a polarized, continuous (i.e., confluent) monolayer of immortalized mammary epithelial cells on the upper surface of the scaffold, wherein the polarized, continuous monolayer comprises an apical surface and a basal surface, thereby producing a live cell construct for producing milk in culture.

21. The method of claim 20, wherein prior to culturing immortalized mammary epithelial cells the method comprises:

(i) isolating primary mammary epithelial cells, myoepithelial cells, and/or mammary progenitor cells from mammary explants from mammary tissue (e.g., breast, udder, teat

tissue), to produce isolated mammary epithelial cells, myoepithelial cells, and/or mammary progenitor cells;

(ii) culturing the isolated primary mammary epithelial cells, myoepithelial cells, and/or mammary progenitor cells to produce a mixed population of primary mammary epithelial cells, mammary myoepithelial cells and mammary progenitor cells;

(iii) sorting the mixed population of primary mammary epithelial cells, myoepithelial cells, and/or mammary progenitor cells to produce a population of primary mammary epithelial cells; and

(iv) stably transfecting one or more cells of the population of primary mammary epithelial cells of (iii) with one or more nucleic acids encoding hTERT or SV40; or transducing with a small hairpin RNA (shRNA) to p16 Inhibitor of Cyclin-Dependent Kinase 4) (p16(INK4)) and Master Regulator of Cell Cycle Entry and Proliferative Metabolism (c-MYC) to produce immortalized mammary epithelial cells.

22. The method of any one of claims 20, wherein the immortalized cell line is stably transfected with one or more nucleic acids encoding hTERT or SV40; or transduced with (a) a small hairpin RNA (shRNA) to p16 Inhibitor of Cyclin-Dependent Kinase 4) (p16(INK4)) and (b) Master Regulator of Cell Cycle Entry and Proliferative Metabolism (c-MYC).

23. The method of any one of claims 19 to 22, further comprising storing the population of primary mammary epithelial cells or the immortalized mammary epithelial cells prior to cultivating on a scaffold, optionally wherein the storing is in a freezer or in liquid nitrogen.

24. The method of any one of claims 17 to 23, wherein the basal surface of the monolayer is adjacent to the upper surface of the scaffold.

25. The method of any one of claims 17 to 24, wherein the live cell construct comprises an apical compartment that is adjacent to the apical surface of the monolayer.

26. The method of any one of claims 17 to 25, wherein the live cell construct comprises a basal compartment that is adjacent to the lower surface of the scaffold.

27. The method of any one of claims 17 to 26, wherein the culturing is carried out at a temperature of about 35°C to about 39°C, optionally about 37°C.

28. The method of any one of claims 17 to 27, wherein the culturing is carried out at an atmospheric concentration of CO_2 of about 4% to about 6%, optionally about 5%.

29. The method of any one of claims 17 to 28, wherein the culturing of (b) comprises culturing in a culture medium that is exchanged about every day to about every 10 days, optionally about every day to about every 3 days.

30. The method of any one of claims 19 to 29, wherein the isolating and sorting is via fluorescence-activated cell sorting, magnetic-activated cell sorting, and/or microfluidic cell sorting.

31. A method of producing milk in culture comprising,

culturing a live cell construct comprising

(a) a scaffold comprising an upper surface and a lower surface and a continuous (i.e., confluent) polarized monolayer of live primary mammary epithelial cells, a continuous polarized monolayer of a mixed population of live primary mammary epithelial cells, mammary myoepithelial cells and mammary progenitor cells, and/or a continuous polarized monolayer of live immortalized mammary epithelial cells having an apical surface and a basal surface, wherein the continuous polarized monolayer of live primary mammary epithelial cells, the continuous polarized monolayer of the mixed population of live primary mammary epithelial cells, mammary myoepithelial cells and mammary progenitor cells and/or the continuous polarized monolayer of live immortalized mammary epithelial cells are located on the upper surface of scaffold,

(b) a basal compartment and an apical compartment, wherein the lower surface of the scaffold is adjacent to the basal compartment and the apical surface of the monolayer of live primary mammary epithelial cells, the monolayer of the mixed population of live primary mammary epithelial cells, mammary myoepithelial cells and mammary progenitor cells, and/or the monolayer of live immortalized mammary epithelial cells is adjacent to the apical compartment,

wherein the monolayer of live primary epithelial mammary cells, the live primary epithelial mammary cells of the monolayer of the mixed population of live primary mammary epithelial cells, mammary myoepithelial cells and mammary progenitor cells, or the monolayer of immortalized mammary epithelial cells excretes milk through its apical surface into the apical compartment, thereby producing milk in culture.

32. The method of claim 31, wherein the basal compartment comprises a basal culture medium and the basal culture medium is in contact with the basal surface of the continuous polarized monolayer of primary mammary epithelial cells, with the basal surface of the continuous polarized the monolayer of the mixed population, or with the basal surface of the continuous polarized monolayer of live immortalized mammary epithelial cells.

33. The method of claim 31 or claim 32, wherein the culturing is carried out at a temperature of about 35°C to about 39°C, optionally about 37°C.

34. The method of any one of claims 31 to 33, wherein the culturing is carried out at an atmospheric concentration of CO_2 of about 4% to about 6%, optionally about 5%.

35. The method of any one of claims 31 to 34, wherein the culturing comprises monitoring the concentration of dissolved O_2 and CO_2.

36. The method of claim 31 to 35, further comprising adding prolactin to the basal culture medium, thereby providing a lactogenic culture medium.

37. The method of any one of claims 31 to 36, wherein the culturing comprises monitoring the glucose concentration and/or rate of glucose consumption in the basal culture medium and/or in the lactogenic culture medium.

38. The method of claim 37, wherein the prolactin is added when the rate of glucose consumption is steady state.

39. The method of any one of claims 36 to 38, wherein the prolactin is produced by a microbial cell or a human cell expressing a recombinant prolactin (e.g., S179D-prolactin).

40. The method of any one of claims 31 to 39, further comprising collecting the milk from the apical compartment to produce collected milk.

41. The method of claim 40, wherein the collecting is via a port.

42. The method of claim 40 or claim 41, wherein the collecting is via gravity or a vacuum, optionally the vacuum is attached to the port.

43. The method of any one of claims 40 to 42, further comprising freezing the collected milk to produce frozen milk and/or lyophilizing the collected milk to produce lyophilized milk.

44. The method of any one of claims 40 to 43, further comprising packaging the collected milk, the frozen milk and/or the lyophilized milk into a container.

45. The method of any one of claims 40 to 42, further comprising extracting one or more components from the collected milk.

46. The method of claim 45, wherein the components from the collected milk are lyophilized or concentrated to produce a lyophilized or a concentrated milk component product.

47. The method of claim 46, wherein the components from the collected milk are concentrated by membrane filtration or reverse osmosis.

48. The method of any one of claims 45 to 47, wherein the lyophilized or concentrated milk component product is packaged in a container.

49. The method any one of claims 45 to 48, wherein the components from the collected milk are milk protein, lipid, carbohydrate, vitamin, and mineral contents.

50. The method of claim 48 or claim 49, wherein the container is sterile.

51. The method of any one of claims 48 to 50, wherein the container is vacuum-sealed

52. The method of any one of claims 48 to 51, wherein the container is a food grade container.

53. The method of any one of claims 48 to 52, wherein the container is a canister, a jar, a bottle, a bag, a box, or a pouch.

53. A method of producing a modified primary mammary epithelial cell or a immortalized mammary epithelial cell, wherein the method comprises introducing into the cell:

(a) a polynucleotide encoding a prolactin receptor comprising a modified intracellular signaling domain, optionally wherein the prolactin receptor comprises a truncation wherein position 154 of exon 10 has been spliced to the 3' sequence of exon 11;

(b) a polynucleotide encoding a chimeric prolactin receptor that binds to a ligand, which is capable of activating milk synthesis in the absence of prolactin;

(c) a polynucleotide encoding a constitutively or conditionally active prolactin receptor protein, optionally wherein the polynucleotide encodes a constitutively active human prolactin receptor protein comprising a deletion of amino acids 9 through 187;

(d) a polynucleotide encoding a modified (recombinant) effector of a prolactin protein comprising (i) a JAK2 tyrosine kinase domain fused to a STAT5 tyrosine kinase domain; and/or (ii) a prolactin receptor intracellular domain fused to a JAK2 tyrosine kinase domain;

(e) a loss of function mutation into a circadian related gene *PER2* (period circadian protein homolog 2); and/or

(f) a polynucleotide encoding one or more glucose transporter genes GLUT1 and/or GLUT12, thereby increasing the rate of nutrient uptake at the basal surface of the monolayer.

55. The method of claim 53, wherein the JAK2 tyrosine kinase domain is fused to the C-terminus of the STAT5 tyrosine kinase domain (e.g., a polynucleotide encoding a JAK2 tyrosine kinase domain is linked to the 3' end of a polynucleotide encoding the STAT5 tyrosine kinase domain).

56. The method of claim 53 wherein the loss of function mutation comprises an 87-amino acid deletion from position 348 to 434 in *PER2*.

ABSTRACT

This invention relates to live cell constructs for producing milk in culture, as well as methods making a live cell construct for producing milk in culture, methods of producing milk in culture, and methods of producing a modified primary mammary epithelial cell or an immortalized mammary epithelial cell for use in a live cell construct and other methods of the present invention.